DOES NOT CIRCULATE
UNIVERSITY OF MICHIGAN
DEC 12
MEDICAL LIBRARY

VOLUME 99 NUMBER 5

BULLETIN of the JOHNS HOPKINS HOSPITAL

THE PUBLICATION OF THE MEDICAL SCHOOL AND HOSPITAL
SUPPORTED BY THE DE LAMAR FUND OF THE JOHNS HOPKINS UNIVERSITY

November, 1956

Effect of Stretch on Oxygen Consumption of Dog Skeletal Muscle in Situ. WENDELL N. STAINSBY, JOHN T. FALES AND JOSEPH L. LILIENTHAL, JR. 249

Effects of Thyroxine on Limb Regeneration in the Newt, Triturus Viridescens. ELIZABETH D. HAY 262

Some Physico-Chemical Aspects of Calcification in Vitro. WILLIAM C. THOMAS, JR., THOMAS B. CONNOR AND JOHN EAGER HOWARD 287

Tubercle Bacillus Bacterial Endocarditis Developed Upon a Surgical Mitral "Sling." R. PATTERSON RUSSELL, MORGAN BERTHRONG AND AUTREY R. CROKE 296

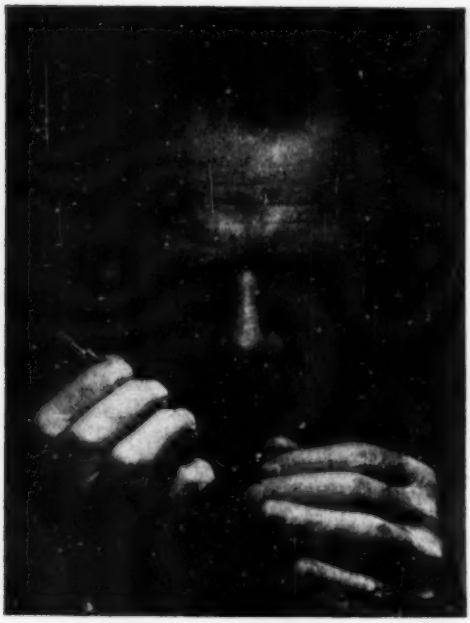
Book Reviews 309

PUBLISHED MONTHLY

THE JOHNS HOPKINS PRESS, Baltimore-18, Maryland

Made in the United States of America

COPYRIGHT, 1956 BY THE JOHNS HOPKINS PRESS



Puts the gouty patient back at work

BENEMID

PROBENECID

When BENEMID was given to gouty workers, immobilized joints showed "...considerable improvement and relaxation so that patients have been returned to useful occupations."[1]

"During the past six years...BENEMID...has demonstrated its value as a uricosuric agent of low toxicity."[2] Indeed, in chronic gout it has been called "the most effective uricosuric substance available.... its use for reduction of size of tophi has been effective."[3]

References: 1. Indust. Med. 22:311 (July) 1953. 2. Journal-Lancet 76:190 (July) 1956. 3. J. Chronic Dis. 2:645 (Dec.) 1955.

MSD

MERCK SHARP & DOHME

DIVISION OF MERCK & CO., INC., PHILADELPHIA 1, PA.

Entered as second class matter at the Baltimore, Md, Post Office. Acceptance for mailing at special rate of postage provided for in Section 1103, act of October 3, 1917. Authorized on July 3, 1918.

EFFECT OF STRETCH ON OXYGEN CONSUMPTION OF DOG SKELETAL MUSCLE IN SITU[1]

WENDELL N. STAINSBY[2, 3], JOHN T. FALES AND JOSEPH L. LILIENTHAL, JR.[4]

The Departments of Environmental Medicine and Medicine, The Johns Hopkins University

Received for publication May 14, 1956

The study to be described herein arose from a chance observation that passive stretch decreased oxygen consumption by the normally-circulated gastrocnemius-plantaris of the dog. The observation arose from studies prefatory to an investigation of the relation between oxygen consumption and isometric work. In order to record maximal isometric tension the muscle must be put under tension, i.e., passive stretch (1). Before accurate measurement of oxygen consumption during isometric work could be made, however, it was necessary to know the effect of passive stretch itself on the resting rate of oxygen use. Feng (2) and Eddy and Downs (3) have reported that extreme stretching of the sartorius muscle of frog increased its heat production, oxygen consumption and carbon dioxide production. In the study to be described it was discovered early that stretch itself caused a marked *decrease* of oxygen consumption. An investigation of this surprising effect of stretch on mammalian skeletal muscle forms the subject of the study.

METHODS AND PROCEDURE

Dogs weighing 7 to 17 kg. were anesthetized with veterinary sodium pentobarbital, 30 mg. (0.45 ml.) per kilogram of body weight, intraperitoneally. Additional doses of 65 mg. were given as needed and at the beginning of each experimental run. The trachea was cannulated and attached to a respirator to maintain adequate ventilation at a constant minute-volume and thus insure a steady oxygen content of arterial blood. Pilot experiments showed that fairly constant arterial oxygen contents were maintained. Arterial samples were taken at ten minute intervals through an indwelling needle in the right femoral artery. In preliminary experiments, simultaneous duplicate samples from the two femoral arteries were analyzed for oxygen content and were found to be identical within the limits of variation of the analytical method; the oxygen content of blood from the right femoral artery was considered therefore, to be that of blood entering the muscle group under study. Mean arterial blood pressure was monitored by means of a mercury manometer attached to the arterial needle. Dogs with blood pressure below 90 mm. Hg were not used.

The muscle group selected for study was the gastrocnemius-plantaris of the left leg. The isolation of the circulation was similar to that of Verzar (4) and Himwich and Castle

[1] Aided by a contract between the Office of Naval Research, Department of the Navy, and The Johns Hopkins University (NR 113–241) and grants from the National Heart Institute (H-1327) and the National Institute for Arthritis and Metabolic Diseases (A-999), National Institutes of Health, Department of Health, Education, and Welfare.

[2] Pre-doctoral Fellow of the National Heart Institute, U. S. Public Health Service.

[3] Present address: National Heart Institute, National Institutes of Health, Bethesda 14, Maryland.

[4] Deceased November 19, 1955.

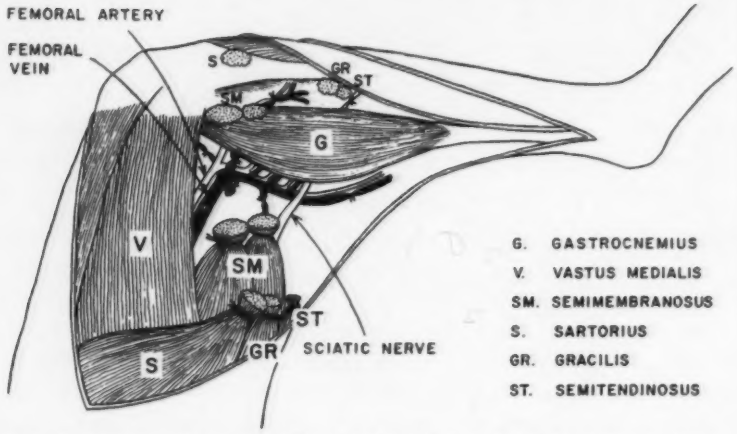


FIG. 1. A diagrammatic representation of the isolation of the circulation to the muscle group. The entire venous outflow was diverted through a cannula at X to the sampling device and flow meter.

(5) and satisfied the following requirements: (a) no interference with arterial inflow, (b) ligation of all tributaries to the femoral vein except those issuing from the muscle group, and (c) ligation of all veins draining the muscle that did not enter the femoral vein directly. To achieve this the following procedure was adopted. From a medial approach the sartorius, gracilis, semitendinosus, and proximal and distal semimembranosus (6) were ligated, divided at their insertions and laid back. The muscle group with its vascular system was thus uncovered. The femoral vein was followed from the entrance of the vein from the sartorius beyond the most distal vein from the muscle group and all tributaries joining it except those from the muscle group were ligated (Fig. 1). All venous channels from the muscle group that did not join the femoral vein were tied. The arterial tree was left untouched.

The femoral vein was then cannulated proximal to the last branch draining the muscle group and connected to a sampling device (Fig. 2A), from which serial samples of venous blood were taken manually into syringes. From the sampler the blood passed to a Gaddum type flow meter similar in design to that described by Green (7) (Fig. 2B), and then to a pump (8) (Fig. 2E), which raised the blood to an infusion bottle (Fig. 2F). The blood was allowed to flow by gravity from the infusion bottle back to the animal through a cannula in the jugular vein.

The common tendon of the muscle group was cut close to the calcaneus and clamped to the pulley system of an isometric tension recording apparatus. Muscle stretch was achieved by hanging weights on the pulley system; lengthening was free. The muscle was kept covered with gauze soaked in warm 0.85 per cent saline, and a warm saline drip was maintained on the muscle during the experiment.

Blood was rendered incoagulable by administration of synthetic heparin-like polysaccharides: Treburon[5] in earlier experiments and Mannuronate[6] in later experiments. Both

[5] Kindly furnished by Dr. E. L. Sevringhaus, Hoffman-LaRoche, Inc., Nutley, New Jersey.

[6] Kindly furnished by Dr. E. F. Roberts, Wyeth Laboratories, Inc., Philadelphia, Pa.

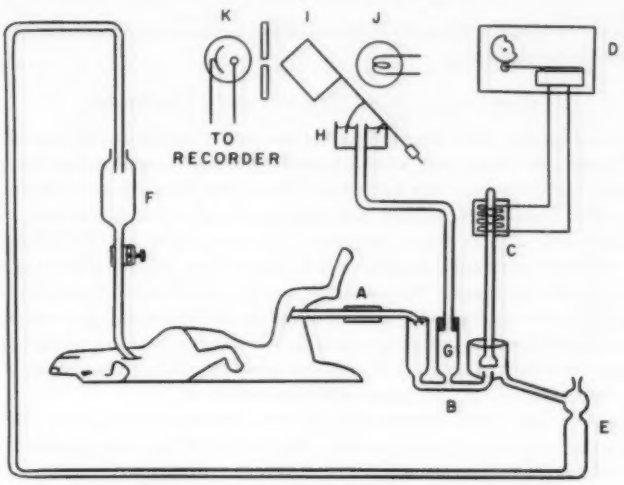


FIG. 2. Diagrammatic representation of the entire extra-corporeal system.

A. Sampling device
B. Flow meter (The ends of the tubing from the femoral vein to the flow meter were always leveled so there was neither positive nor negative pressure at the cannula)
C. Solenoid operated valve
D. Synchronous motor, cam and microswitch operating the solenoid
E. Pump
F. Infusion bottle
G. Side of flow meter "U" tube connected to the volume recorder
H. Volume recorder
I. Vane on the volume recorder
J. Light source
K. Photocell

agents proved to be adequate when an initial dose of 50 mg. per kilogram of body weight and then 100 mg. every half hour were injected intravenously in aqueous solutions of 250 mg./ml. and 100 mg./ml. respectively. Except for the rubber connectors at the two cannulae, all extra-corporeal blood channels were made of Tygon or glass which had been treated with silicone (G. E. "Dri-Film"). On some occasions the plasma was very faintly stained with hemoglobin at the end of the experiment; in most cases plasma remained water-clear.

The dead space of flow meter, pump, infusion bottle and connecting tubes required that about 125 ml. of blood be extra-corporeal. To avoid this significant loss of blood volume the system was filled with 6 per cent dextran in 0.85 per cent sodium chloride (Expandex, Commercial Solvents Corporation) prior to cannulation. As soon as venous outflow from the muscle group was diverted through the cannula to the flow meter, dextran was allowed to enter the jugular vein at the same rate. Thus, changes in circulating blood volume were kept at a minimum. About twenty minutes were allowed for mixing of dextran in the circulating blood volume before sampling was begun. Throughout the remainder of the experi-

ment the return of blood to the animal was regulated by an adjustable clamp to balance the outflow from the muscle group.

Handling of Blood Samples and Calculations

Venous blood samples were drawn from the sampler at approximately ten minute intervals when the muscle was at rest. During periods of change venous samples were taken as often as every two minutes or less as desired. During short periods when there were rapid changes of blood flow the entire flow was collected under liquid petrolatum and aliquots taken for calculation of mean oxygen consumption for the period. For A-V oxygen difference the oxygen content of the venous samples was subtracted from oxygen content of the chronologically closest arterial sample. The collecting system introduced a dead space of the order of 1.2 ml. between the muscle and the site of sampling. To insure that any sample of venous blood was assigned its proper position in time and in relation to the blood flow recorded instantaneously the approximate delay time for the blood to arrive at the sampler after leaving the muscle was calculated for all ranges of flows encountered.

Arterial and venous blood samples, 0.6 ml., were drawn into 1 ml. tuberculin syringes, the dead space of which had been previously filled with 0.85 per cent sodium chloride. The amount of sodium chloride required to fill the dead space diluted the sample of blood about 1 per cent, with an insignificant variation from one syringe to another. No correction was made for this small dilution. Immediately after each sample was drawn it was sealed with a cap containing mercury, a portion of which was introduced into the syringe to provide a means of mixing. Samples were kept iced until further handling, and prior to analysis were mixed by rotation in an icewater bath for at least ten minutes. Blood was then hemolyzed by anaerobic transfer to other tuberculin syringes, the dead spaces of which had been previously filled with a partially saturated solution of saponin,[7] 6.25 g. per 100 ml. After hemolysis the blood was transferred to a Nahas cuvette, and its optical density determined against a plasma-saponin blank at 605 and at 505 mμ in the Beckman Model DU spectrophotometer, as described in a previous paper (9).

The rate of oxygen consumption was calculated in μl./g. of muscle per minute by substituting into the Fick equation:

$$\dot{Q}_{O_2} = \frac{F(A - V)}{W} \times 1000,$$

where $\dot{Q}_{O_2}$ = the rate of oxygen consumption in μl./g./min.; F = total muscle blood flow in ml./min.; A and V = oxygen content of 1 ml. of arterial and venous blood samples respectively; and W = weight of the muscle in grams. For measurement of changes in oxygen consumption the observed values for resting oxygen consumption were extrapolated through the experimental period. Oxygen debt and repayment were calculated by measurement of the area between the extrapolated resting rate and the observed rate of oxygen uptake.

RESULTS

Oxygen Consumption at Rest

In ten experiments (Table II), during rest prior to stretch, mean muscle oxygen consumption was 4.9 μl./g./min. (range 2.4–9.0). In an additional three experiments oxygen consumption was measured for one and one-half hours with muscle at rest to determine whether or not there were changes with time. No such changes were found (Fig. 3A).

[7] Eastman, Practical, Eastman Kodak Company, Rochester, New York.

TABLE I

Protocol of a Sample Experiment

Experiment No. 94 Dog weight: 17 kg., Muscle weight: 74.6 g., Stretch: 7.54 kg.

Time min.	Sample Number		Arterial O_2 Vols. p.c.	Venous O_2 Vols. p.c.	A-V O_2 ml./min.	Blood Flow ml./min.	Total O_2 Consumption	
							ml./min.	μl./g./min.
0	A_1*		19.3					
1		V_1†		12.7	.066	4.8	.316	4.2
10	A_2	V_2	18.9	15.4	.035	6.3	.221	3.0
20	A_3	V_3	19.6	15.3	.043	6.4	.270	3.7
28	A_4		20.0					
29		V_4		15.2	.047	6.8	.321	4.3
30	Stretch on							
35		V_5		17.1	.021	3.7	.077	1.1
39	A_5		19.2					
40		V_6		15.4	.037	3.2	.121	1.6
45		V_7		15.9	.037	3.6	.126	1.7
49	A_6	V_8	19.6	15.1	.044	3.2	.141	1.9
50	Stretch off							
51		V_9		16.0	.035	10.9	.384	5.2
53		V_{10}		13.8	.062	6.6	.408	5.5
54	A_7		20.0					
55		V_{11}		13.1	.069	5.8	.399	5.4
65	A_8	V_{12}	19.4	13.6	.059	5.8	.342	4.6
74	A_9		19.3					
75		V_{13}		13.0	.063	5.2	.328	4.4
86	A_{10}	V_{14}	19.3	12.0	.073	4.4	.320	4.3

* A = Arterial

† V = Venous

TABLE II

Summary of Stretch Results

Expt. no.	Body Weight kg.	Muscle Weight g.	Stretch* Tension kg.	Delta A-V O_2 (ml./min.)		Blood Flow (ml./min.)		(S/R) 100	O_2 Consumption (μl./g./min.)		
				Range	Av.	Range	Av.		Range	Av.	(S/R) 100
31	12.0	43.0	2.3	R 8.9–9.0	9.0	4.2	4.2	48	8.6–8.8	8.7	55
				S 9.8–10.9	10.2	1.7–2.5	2.0		3.9–6.3	4.8	
39	7.3	26.0	2.0	R 6.9–7.7	7.3	2.4–2.5	2.4	30	6.4–7.2	6.8	32
				S 7.2–8.9	8.0	0.7–0.8	0.7		1.9–2.4	2.2	
71	7.6	25.2	4.0	R 2.5–5.1	3.5	2.2–3.6	2.9	28	3.5–4.5	3.8	36
				S 3.2–5.7	4.4	0.7–0.9	0.8		1.0–1.7	1.4	
78	10.5	45.0	4.0	R 2.0–2.8	2.4	5.5–6.8	6.2	42	3.0–3.7	3.3	60
				S 3.0–5.1	3.7	2.0–3.3	2.6		1.9–2.6	2.0	
83	9.5	39.8	4.0	R 8.8–11.0	9.9	2.2–3.5	2.7	34	6.0–9.0	7.4	30
				S 6.7–12.0	10.1	0.6–1.3	0.9		1.8–2.9	2.2	
84	17.0	69.0	6.0	R 2.5–4.1	3.6	10.4–17.4	13.6	37	5.7–7.8	6.6	65
				S 4.7–7.6	6.1	3.8–6.6	5.0		3.9–4.5	4.3	
85	11.2	33.0	4.0	R 5.9–7.5	6.7	1.4–2.2	1.8	24	3.0–4.6	3.7	27
				S 9.2–10.3	9.6	0.3–0.4	0.4		0.4–1.4	1.0	
92	12.5	57.5	6.0	R 4.5–4.8	4.7	3.2–3.4	3.3	66	2.6–2.8	2.7	85
				S 5.8–6.1	6.0	2.0–2.2	2.2		2.1–2.4	2.3	
94	17.0	74.6	7.5	R 3.5–6.6	4.8	4.8–6.8	6.1	56	3.0–4.3	3.8	41
				S 2.1–4.4	3.5	3.2–3.7	3.4		1.1–1.9	1.6	
111	9.0	37.0	5.5	R 4.0–5.2	4.4	2.0–2.2	2.2	38	2.4–2.8	2.6	42
				S 2.8–6.5	4.9	0.8–0.8	0.8		0.6–1.5	1.0	

R = Resting

S = Stretch

* Duration of stretch was 20 minutes except in Expt. 31 in which it was only 5.

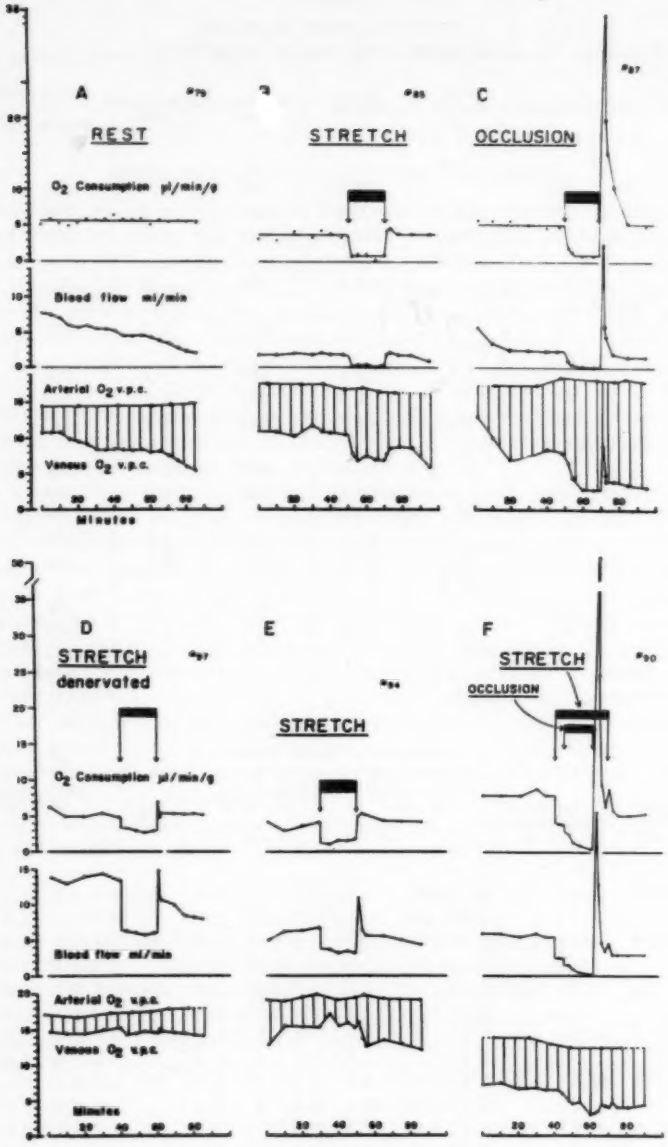


FIG. 3

A = Arterial and venous oxygen contents, blood flow and oxygen consumption of the muscle group at rest
B = Effect of twenty minutes of stretch
C = Effect of twenty minutes of occlusion
D = Effect of twenty minutes of stretch on a denervated muscle group
E = Effect of twenty minutes of stretch
F = Effect of occlusion during stretch

Effects of Stretch

Steady stretch at a tension of approximately 100–200 g./g. of muscle produced invariably an immediate sustained fall in oxygen consumption (Fig. 3B and E and Table I). The larger part of the fall in oxygen consumption was accounted for by concomitant fall in blood flow since A-V oxygen difference changed only slightly. On the average, oxygen consumed during stretch was 47 per cent of that at rest (range 27–85 per cent). Average oxygen debt during 20 minutes of stretch was 42 μl./g. of muscle (range 7.6–72.6) (Table II). No relation between amount of tension and fall in oxygen consumption was apparent. In an additional three experiments, not recorded in Table II, tensions were between 75 and 100 g./g. and there was no fall in oxygen consumption. This does not necessarily demonstrate a threshold phenomenon since in three of the experiments recorded in Table II tensions were equally low.

On release of stretch blood flow and oxygen consumption returned promptly to pre-stretch levels with a slight transient overshoot. This overshoot accounted for 10.6 per cent of the oxygen debt, on the average, and on one occasion for as much as 23 per cent (Table I, Fig. 3B and E).

Essential to the interpretation of all experiments to be described is the certain knowledge that all blood flowing through the muscle under study issued from the cannulated vein. In the event that, under conditions of stretch, increased intramuscular pressure diverted blood away from the collecting site, (e.g., into the femur) then the measurement of blood flow would be in error by as much as disappeared into other areas. To test this possibility the experimental muscle group was injected at several sites with a total of 3 ml. of 1 per cent fluorescein in mammalian Ringer's solution. While the muscle was stretched the venous outflow through the femoral vein was diverted and did not return to the general circulation for a period of 15 minutes; this blood contained large amounts of fluorescein. During this period samples of blood were drawn from the general circulation and examined under ultraviolet light for the presence of fluorescein. None was detected; and by serial dilution the level of detection was determined to be such that fluorescein would have been identified readily if no more than 0.5 per cent of the total injected dye had escaped into the general circulation.

Effects of Stretch on Acutely Denervated Muscle

To test whether the reduction of oxygen consumption depended upon central reflexes, a series of three experiments was done with the muscle group completely denervated by clearing the artery and vein of all connective tissue and cutting the sciatic nerve. The distal part of the cut nerve was blocked with 2 per cent procaine prior to section to prevent spontaneous nerve impulses arising at the site of injury from reaching the muscle group. It was observed in preliminary studies that the increased rate of flow following denervation

declined to some extent with time. To allow as much time as possible for the flow to return to normal, the nerve was cut as soon as possible during the preparatory part of the experiment. The usual lapse of time from nerve section to the beginning of the collection of data was two hours. Even then the blood flow was high and the resulting A-V difference was so small that the analytical errors in the blood oxygen content determinations became significant. The result was a greater apparent variation in the resting rate of oxygen consumption. In one of the three experiments these variations were so great that reliable calculations were impossible. In the other two experiments the oxygen consumption fell during stretch to 56 and 57 per cent of the resting rate respectively, and there was no payment of the debt (Fig. 3B and E).

Effects of Arterial Occlusion

The pinnate arrangement of the muscle fibers and their attachments is such that pull on the tendon almost surely increases hydrostatic pressure within the muscle. Increased hydrostatic pressure might have completely occluded the flow through some areas of the muscle. Thus, the oxygen consumption of the muscle would be reduced by the amount of muscle that was not being perfused. To test whether occlusion of flow would produce an oxygen consumption pattern resembling that of stretch, four experiments were done in which the entire flow to the muscle group was slowed or stopped by a tourniquet around the upper thigh. Occlusion was maintained for the same length of time as stretch, i.e., 20 minutes. Oxygen consumption was followed in the same manner except during complete occlusion when there was no flow. When occlusion was released there was an immediate reactive hyperemia and increased oxygen uptake. Measurements of areas under the respective curves showed that the volume of extra oxygen taken up after occlusion was at least equal to the debt incurred during occlusion (Fig. 3C).

Absence of Muscular Activity During and After Occlusion

It will be noted that although stretch and occlusion both produced decreased oxygen consumption the two differed strikingly in that there was little or no debt repayment following release of stretch. One conceivable explanation for increased oxygen consumption and apparent debt repayment following release of occlusion was that increased muscular activity may have been stimulated by release of occlusion since such increased activity has been observed following 30-minutes occlusion of the human forearm (10). To test this possibility, bipolar electrodes were placed in the muscle group of the dog and muscle action potentials were displayed on a cathode ray oscilloscope. There were no action potentials in the preparation at rest, during 20 minutes of occlusion or following occlusion. Thus it would seem that increased muscular

activity cannot account for the increased oxygen consumption following occlusion.

Effect of Occlusion During Stretch

In two experiments flow to the leg was occluded during stretch. After a period of measurement of oxygen consumption at rest the muscle was stretched for ten minutes. Blood flow to the leg was then occluded for fifteen minutes while muscle stretch continued. Release of occlusion during stretch resulted in hyperemia and increased oxygen uptake similar to that when there was no stretch (Fig. 3F). After recovery from occlusion stretch was released and oxygen consumption returned to the previous resting rate. It was observed that the amount of extra oxygen taken up after the occlusion was released was but slightly larger than the debt produced during occlusion and was smaller than the total debt incurred during stretch plus occlusion. This observation indicated that stretch did not prevent increased blood flow and oxygen uptake induced by ischemia.

DISCUSSION

Viewed in broadest terms, oxidative metabolism supplies energy for synthesis and repair, transport of substances against concentration gradients and muscular contraction. It is conceivable that the inactive contractile mechanism requires energy for maintenance in a state ready to contract. This stand-by function plus maintenance of concentration gradients across various membranes may indeed account for a large part of the energy requirement of resting muscle.

Oxygen consumption of resting muscle may vary with changes in energy expenditure of the muscle, or with metabolic shifts to pathways that utilize more or less oxygen to meet constant energy requirements, i.e., changes in the efficiency of utilization of oxygen or a combination of these two. In the first instance one can visualize changes in energy expenditure which would or would not be reflected in the rate of oxygen consumption depending on the direction of a simultaneous change in the efficiency of oxygen utilization. Since the preparation did not provide data susceptible to an approximate thermodynamic analysis, these possibilities remain unevaluated.

What other explanations may account for the fall in oxygen consumption that accompanies stretch? The phenomena that have been considered are that the hypometabolism during stretch is (1) apparent only and not real, (2) the result of lowered partial pressure of oxygen (P_{O_2}) in the muscle tissue, and (3) owing to an actual fall in cellular requirement.

1. Three possibilities that might create an apparent fall in oxygen consumption during stretch are errors in blood flow measurement, utilization of oxygen stores in the muscle, and locally incomplete oxidation which is completed at another site.

If part of the venous outflow from the muscle were diverted into other areas and not into the flow meter the result, discussed above, would be a fall in blood flow with no change in A-V oxygen difference and a consequent fall in the computed oxygen consumption. However, the fluorescein experiments make it apparent that the entire venous drainage from the muscle was in fact measured.

All muscles contain a certain amount of myoglobin which can release stored oxygen when the tissue P_{O_2} falls. If these stores were large enough the muscle could utilize the oxygen from them during stretch thus extracting less oxygen from blood perfusing the muscle and thereby creating an apparent decrease in the rate of oxygen consumption as measured by A-V difference and flow. Although no data are available on the storage capacity of dog muscle there are relevant data on the oxygen storage of cat soleus muscle (11). The calculated storage capacity for cat soleus is 6.6 μl./g. If this capacity is assumed to describe the approximate storage capacity of similar muscles in the dog, then it would appear that the average oxygen debt from 20 minutes of stretch (42.1 μl./g.) is some six times more than can be accounted for by utilization of stores. Furthermore, if provision of oxygen from stores accounted for a normal consumption in the face of an apparent decrease, as described by Millikan for the cat soleus, a period of heightened consumption should appear. This, in fact, did not occur.

When oxygen supply is reduced muscle is capable of supplementing its energy production by anaerobic metabolism producing lactate to be metabolized at another site. The oxygen consumption in muscle then would be reduced by that portion of the oxidative metabolism that was completed elsewhere. In two preliminary experiments, muscle lactate production was estimated by measurement of blood flow and lactate A-V difference. Although there appeared to be some increase in lactate production during and after stretch, calculation based on standard values for complete combustion of glucose and of lactate did not support the possibility that more than about 10 per cent of the total energy debt could have been met by anaerobic glycolysis in these two experiments.

2. There are observations which have been interpreted to indicate that lowered P_{O_2} in muscle tissue results in lowered oxygen consumption, either by restricting blood flow or by reducing the capillary diffusing surface (12, 13, 14, 15). That lowering muscle P_{O_2} is unlikely to be the explanation of the stretch phenomenon is indicated by the following relationship. From the Fick equation it can be seen that in meeting a constant oxygen requirement when the arterial P_{O_2} is constant, the venous P_{O_2} must fall as the flow decreases. Owing to the relation between blood and tissue oxygen, a fall of the tissue P_{O_2} must be reflected in the P_{O_2} of venous blood. It has been demonstrated in a somewhat different experimental preparation that as P_{O_2} falls below 50 to 55 mm.

Hg oxygen transport declined and oxygen consumption decreased (16). By contrast in the present preparation resting oxygen consumption remained unchanged even when the P_{O_2} of venous blood fell to an estimated 20 mm. Hg (Fig. 3A). However, if arterio-venous anastomoses exist in muscle, a possibility that has been proposed (12) but never demonstrated anatomically (17), then if tissue P_{O_2} were indeed low during stretch this change would have been obscured by mixing of hypoxic blood from muscle cells with arterial blood which had passed through shunts. The occlusion experiments verify the observation that when the oxygen consumption falls during a period of inadequate supply of oxygen, the deficit is repaid when the oxygen is again supplied in sufficient quantity (18). This is not so with stretch, and it would seem that some factor other than uniform lowering of P_{O_2} of the tissue caused the fall in oxygen consumption.

Another explanation of the fall in oxygen consumption during stretch is based on the possibility that oxygen consumption of muscle is determined by supply, and that within certain limits the oxygen consumption at any given time is a result of the number of functional capillaries and the P_{O_2} and flow rate of the blood passing through them. If, when the muscle is stretched, portions of the capillary bed were partially or completely occluded, flow through these vessels might be slowed or stopped, decreasing the availability of oxygen to the parts of the muscle that they supply. Under these conditions the oxygen consumption would fall during stretch and then return to the previous resting rate with no debt repayment, because flow returned only to the previous level. By contrast, following occlusion there is reactive hyperemia, a high flow through more capillaries, with a resultant increase in oxygen consumption. The fact that the excess oxygen consumption after occlusion was equal to the debt created during occlusion may then be the result of the fact that the flows were equal. This explanation of the decreased rate of oxygen consumption during stretch is applicable to the decreased rate of oxygen uptake during sympathetic activity (12, 13, 14, 15), the diving reflex (19), lowered blood flow (18) and lowered arterial P_{O_2}. If the oxygen consumption of muscle is supply dependent then in view of the stability of oxygen consumption during large changes of blood flow in resting muscle, there must be an excellent regulation of the distribution of flow through metabolizing and non-metabolizing areas, and this, in turn, presupposes A-V anastomoses in muscle.

3. It is possible that there is a real lowering of the metabolic needs of muscle cells while they are stretched. The speculative assumption is made here that energy is spent in keeping the contractile proteins in their partially extended state. If part of this energy were now furnished by an external source, stretch, it is possible that the saving to the internal source would be reflected in a lowered rate of oxygen consumption. It is difficult to align with this hypothesis the observed fact that the fall of oxygen consumption does not occur consist-

ently below a critical level of stretching force. However, a further assumption may be made that at and above a critical degree of stretch a sudden rearrangement, slip or extension of the protein chains may occur to a new configuration which requires less energy. The fact that the resting length tension relation is determined by the sarcolemma (1, 20) may make it misleading to quantitate the amount of stretch in terms of force, since the critical factor may be the actual length of the muscle fibers.

Another proposal envisions an effect of stretch on the properties of the muscle membrane itself. It has been shown that both sodium efflux and excitability of frog muscle are altered by passive stretch (21, 22), suggesting that stretch itself may have profound effects on processes requiring energy for maintenance.

Stimulation of sympathetic nerves may reduce oxygen consumption as a response of muscle to impulses from certain nerve fibers. Stretch may trigger the same mechanism locally by axone reflex. The possibility exists, since autonomic fibers alter metabolism in secretory cells, that they may have an effect on muscle as well. Although anatomical evidence does not favor this possibility and there is no evidence other than the response to sympathetic activity previously reported (12, 13, 14, 15) there is no evidence that definitely excludes this possibility.

SUMMARY

When the gastrocnemius-plantaris muscle group of the dog was stretched by tensions slightly greater than 100 g. per g. of muscle the rate of oxygen consumption fell on the average to half of the resting rate and remained at this low level until tension was released. When the tension was released the oxygen consumption returned to the previous resting rate with little payment of the oxygen debt created during stretch. In the preparation studied the rapid and complete repayment of the oxygen debt following complete or partial occlusion was not observed following stretch. It would seem that stretch reduced oxygen consumption by some mechanism other than relative anoxia due to generalized circulatory interference.

REFERENCES

1. Ramsey, R. W. and Street, S. F.: The isometric length tension diagram of isolated skeletal muscle fibers. J. Cell. Comp. Physiol. 1940, **15:** 11.
2. Feng, T. B.: The effect of length on the resting metabolism of muscle. J. Physiol. 1932, **74:** 441.
3. Eddy, N. B. and Downs, A. W.: Extensibility of muscle: the production of carbon dioxide by a muscle when it is made to support a weight. Am. J. Physiol. 1921, **56:** 188.
4. Verzar, F.: The gaseous metabolism of striated muscle in warm blood animals. Part I. J. Physiol. 1912, **44:** 243.
5. Himwich, H. E. and Castle, W. B.: The respiratory quotient of resting muscle. Am. J. Physiol. 1927, **83:** 92.

6. ELLENBERGER, W. AND BAUM, H.: *Anatomie des Hundes*. P. Parey, Berlin, Germany, 1891.
7. GREEN, H. D.: Venous drainage recorders. In *Methods in Medical Research*. Vol. I. The Year Book Publishers, Inc., Chicago, Illinois, 1948.
8. ANDERSON, F. F. AND CRAVER, B. N.: Compact and efficient apparatus of pyrex glass for coronary perfusion. Fed. Proc. 1947, **6**: 1.
9. STAINSBY, W. N., FALES, J. T. AND LILIENTHAL, J. L., JR.: A rapid spectrophotometric method for determining oxygen content of dog's blood. J. Appl. Physiol. 1955, **7**: 577.
10. MAGLADERY, J. W., MCDOUGAL, D. B., JR. AND STOLL, J.: Electrophysiological studies of nerve and reflex activity in normal man. III. The post-ischemic state. Bull. Johns Hopkins Hosp. 1950, **86**: 313.
11. MILLIKAN, G. A.: Experiments on muscle hemoglobin *in vivo*, the instantaneous measurement of muscle metabolism. Proc. Roy. Soc. London, England, 1937, B **123**: 218.
12. PAPPENHEIMER, J. R.: Vasoconstrictor nerves and oxygen consumption of isolated hind limb of the dog. J. Physiol. 1941, **99**: 182.
13. NAKAMURA, H.: The oxygen use of muscle and the effect of sympathetic nerves on it. J. Physiol. 1921, **55**: 100.
14. REIN, H. AND SCHNEIDER, M.: Die lokale Stoffwechseleinschränkung bei Reflektorisch—Nervöser Durchblutungsdrosselung. Pflüger's Arch. 1938, **239**: 464.
15. BÜCHERL, E. AND SCHWAB, M.: Der Sauerstoffverbrauch des ruhenden Skeletmuskels bei Reflektorisch—Nervöser vasokonstriktion. Pflüger's Arch. 1952, **254**: 337.
16. PAPPENHEIMER, J. R.: Blood flow, arterial oxygen saturation and oxygen consumption in the isolated hind limb of the dog. J. Physiol. 1941, **99**: 283.
17. BOYD, J. D.: A general survey of vascular structures. In *Visceral Circulation*. Little, Brown and Co., Boston, Mass., 1953.
18. REIN, H. AND SCHNEIDER, M.: Die answerkung künstlicher Mangeldurchblutung auf den lokalen Stoffwechsel. Pflüger's Arch. 1938, **239**: 451.
19. SCHOLANDER, P. F.: Experimental investigations on the respiratory function in diving mammals and birds. Hvåldarets Skrifter Norske Vidanskaps-Akad. i. Oslo, 1940, No. 22.
20. BUCHTHAL, F. AND STEN-KNUDSEN, O.: *The Muscle, A Study in Biology and Pathology*. (Ed. Schapira) l'Expansion Scientifique Française, Paris, France, 1952.
21. PUPILLI, G. C.: Changes of excitability of frog skeletal muscle from passive stretch. Arch. sc. biol. 1945, **30**: 282.
22. HARRIS, E. J.: An effect of stretch upon sodium output from frog muscle. J. Physiol. 1954, **124**: 242.

EFFECTS OF THYROXINE ON LIMB REGENERATION IN THE NEWT, TRITURUS VIRIDESCENS

ELIZABETH D. HAY

Department of Anatomy, The Johns Hopkins University School of Medicine

Received for publication May 18, 1956

The present study of the effect of hyperthyroidism on limb regeneration in the salamander stems from an interest in factors which cause animals to lose this remarkable regenerative capacity. The frog tadpole loses the ability to regenerate a limb at the time of metamorphosis and, since thyroid hormone brings about metamorphosis, it has been natural to speculate that this hormone might play a rôle in the loss of this ability (see review by Lynn and Wachowski (1)). Thyroid and pituitary preparations have been given to tadpoles and studies made of limb and tail regeneration (2–10) with various results. The effects of thyroid on limb regeneration in the tadpole (inhibition if thyroid is administered before amputation and acceleration if thyroid is given after the regenerate has begun to grow (6, 10)) are related to, and may be secondary to, the effects on metamorphosis of the preparation. In order to determine if thyroid itself has any direct effect on regeneration, we chose to administer the hormone to the adult salamander because this amphibian retains the power to regenerate its limbs after metamorphosis. Previous investigations (11, 12) suggest that thyroid has a retarding effect on limb regeneration in the salamander. Negative results have been reported (13, 14), which may be due to low dosage or treatment begun too late in regeneration. The present study is the first of a series to analyse more thoroughly the action of thyroid on limb regeneration in the salamander. It is the object of these experiments to explore the effects of different concentrations of thyroxine given at different times during regeneration.

MATERIALS AND METHODS

The animals used in the experiments to be reported were aquatic adults of the species *Triturus viridescens viridescens* (common spotted newt). They were collected locally or obtained from a supply house (Turtox). Sodium L-thyroxine pentahydrate (*Synthroid*, contributed by Travenol Laboratories) was used in the majority of the experiments. Thyroxine crystals (Squibb) and desiccated thyroid (Squibb) were also employed briefly. The newts were kept in glass or plastic containers holding 4000 ml. of water and 10 animals each. Individual animals were identified by charts of their pigment patterns. The appropriate amount of a fresh stock solution of thyroxine (10 mg. in 95 ml. of distilled water and 5 ml. of 1 per cent NaOH) was added to the water in which the newts were kept each time it was changed (every other day). The concentration $1 \cdot 10^{-8}$ had no effect on molting in preliminary experiments (molting is one index of thyroid activity in *Triturus* (15)). The concentrations $1 \cdot 10^{-7}$ and $1 \cdot 10^{-6}$, which increase molting, were used in the experiments to be re-

TABLE I

Summary of the Experiment[1]

Treatment	Exp. No.	Source of Newts	Time of Treatment	Date Rx Begun	No. of Animals Alive at:					
					Beg.	Amp.	3 wk.[2]	5 wk.	7 wk.	12 wk.
Group A. Continuous Treatment										
$1 \cdot 10^{-7}$ thyroxine	5(1)	G.B.S.	Begun 1 mo. pre-amp.	1/54	21	17	11	11	8	6
	6(1)	G.B.S.	Begun 2 mos. pre-amp.	1/54	21	11	7	6	6	*
Desic. Thyroid	7(1)	Local	Begun 1 wk. pre-amp.	3/54	15	15	15	11	8	*
	7(2)	Local	Begun 1 mo. pre-amp.	3/54	15	15	9	8	*	*
$1 \cdot 10^{-7}$ thyroxine followed by $1 \cdot 10^{-6}$ thyroxine	5(2)	G.B.S.	10^{-7} begun at amp. 10^{-6}, 5 wk.	2/54	19	19	13	12	7	0
	6(2)	G.B.S.	10^{-7} for 5 wks. pre-amp. 10^{-6} at amp.	2/54	19	11	4	2	0	*
$1\ 10^{-6}$ thyroxine	7x(1)	Local	Begun 3 wks. post-amp.	4/54	5	5	5	5	2	0
	7x(2)	Local	Begun at amp.	4/54	5	5	4	0	*	*
Group B. Discontinuous										
$1 \cdot 10^{-6}$ thyroxine for 5 wks. tapered for 3 wks. then discontinued	8(1)	Local	Begun 9 das. pre-amp.	7/54	19	19	18	4	2	2
	8(2)		Disc. 2 wks. pre-amp.	7/54	2	2	2	2	2	2
	8(3)	Local	Disc. 6 mos. pre-amp.	7/54	2	2	2	2	2	1
$1 \cdot 10^{-6}$ thyroxine for 2 wks.	4(1)	G.B.S.	Begun at amp.	9/53	16	16	8	8	7	7
$1 \cdot 10^{-6}$ thyroxine for 2 wks. tapered for 1 wk.	4(2)	G.B.S.	Begun 3 wks. post-amp.	9/53	16	16	16	12	7	6

[1] The controls are not shown in this table (see text). Date Rx begun is given as month/year and refers to the time treatment was begun. Abbreviations: amp.—amputation; beg.—beginning; desic.—desiccated; disc.—discontinued; G.B.S.—General Biological Supply (Turtox); *—experiment discontinued.

[2] Time is given here as weeks after amputation.

ported. (These concentrations readily produce metamorphosis in the tadpole.) Desiccated thyroid was fed as a mush mixed with liver by stomach tube, one 16 mg. tablet (0.2 per cent iodine) per newt three times a week (experiment 7).

Animals were fed liver, ground beef or shrimp individually by forceps three times a week. The hyperthyroid newt has a poor appetite and loses weight, but starvation of controls did not affect regeneration and no attempt was made to withhold food, since starved newts frequently bite each other's regenerates. In preliminary experiments (1951–52), various antibiotics and vitamins, different diets, boiled water and spring water, and cooler temperatures were tried, but no way was found to prevent the frequent deaths (Table I) of animals

receiving thyroxine. Large containers of tap water with green water plants to prevent fungus growth proved to be the healthiest environment. The continuous molting and poor condition of the skin suggested that infection via the skin might have caused some of the deaths, but vital organs might have been affected. Taylor (16) noted small, white spleens in salamanders dying after receiving thyroid transplants, but we did not find any striking abnormalities in the organs of treated animals on gross examination.

Amputations were through the middle of the radius and ulna in all of the experiments to be reported. Camera lucida drawings of the ventral aspect of the regenerating limb or limbs of every animal were made at weekly intervals for the first 7 weeks of regeneration and at less frequent intervals later (usually at 9, 12, 15 and 20 weeks). Where measurements of the size of the regenerates were made, a planimeter was used to obtain the area of the camera lucida drawing of the regenerate. For the purposes of reporting the observations, the following terms are used for regeneration stages in this report: (1) early blastema, comprising all changes prior to the onset of visible growth through the early bud stage (Singer, (17)), (2) cone stage (medium bud, (17)), at which time the proliferating blastema becomes conical in shape, (3) paddle stage, which is characterized by further proliferation, distal flattening of the regenerate and internal signs of early differentiation, (4) three toes, which first appear as small buds, (5) 4 toes, the final stage. The regenerate continues to grow for at least 6 months after the final toe makes its appearance. When the amputation level is through the

TABLE II

Effect of Thyroid on the Rate of Appearance of the Initial Stages of Regeneration[1]

Exp. No.	Treatment	3 Wks. after Amp.					4 Wks. after Amp.					5 Wks. after Amp.				
		E.B.	Cone	Pad.	3 T.	4 T.	E.B.	Cone	Pad.	3 T.	4 T.	E.B.	Cone	Pad.	3 T.	4 T.
5(1)	$1 \cdot 10^{-7}$ from preamp.	6	3	2				3	4	4				5	3	3
5(2)	$1 \cdot 10^{-7}$ from amp.	6	5	2				3	4	5				3	4	5
5C	Controls	3	8	2					6	5	1			1	3	8
6(1)	$1 \cdot 10^{-7}$ from preamp.	2	1	3	1			1	2	2	1			1	4	1
6(2)	$1 \cdot 10^{-6}$ from amp.	1	3				1	1	1			1			1*	
6C	Controls		2	6	2			1	2	3	4			2	1	7
7z(1)	$1 \cdot 10^{-6}$ from 3 wks.		1	3	1				1	3	1			1	2	2
7(1)	Desic. thyroid	3	8	3	1			2	5	5	1			3	3	5
7C(1)	Controls	1	4	4	1				4	6				1	3	6
7z(2)	$1 \cdot 10^{-6}$ from amp.	4					2									
7(2)	Desic. thyroid	7	1	1				3	4	1				3	4	1
7C(2)	Controls	2	4	4				1	3	6					4	5
8(1)	$1 \cdot 10^{-6}$ from preamp.	9	4	4	1			1	5	2*				2*		2*
8C(1)	Controls		2	2	5					1	8					9
4(1)	$1 \cdot 10^{-6}$ to 2 wks.	7	1					3	5					2	4	1
4C	Controls	5	4	4				1	1	7	3				5	6

[1] For each experiment listed, the number of regenerating limbs in each stage is shown at 3, 4 and 5 weeks after amputation. E.B.—early blastema. Pad.—paddle. T.—toes. *—stunted, abnormal regenerates. See Table I for further explanation of treatment and abbreviations.

forearm, the cone usually forms about two to three weeks after amputation, the paddle several days later and the toebuds 4–5 weeks after amputation. In one experiment regeneration was more rapid (8(1), Table II), although the temperature was the same (23°C.; A constant temperature box was used). These animals were collected locally in a drying pond in the summer and were unusually vigorous. After they had been in the laboratory for three months, their limbs regenerated at the normal rate.

This paper deals with 234 regenerating limbs, of which 81 were controls, and each of which was followed individually with camera lucida drawings. These limbs are divided into a series of experiments (Table I). With the exception of experiment 4(2) which will be described later, the control and experimental newts had their limbs amputated at the same time and were kept in the same kind of containers and at the same temperature (approximately 23°C.) in each experiment. In every experiment, the control and experimental newts were from the same source and had been in the laboratory the same length of time. It was found important to control all of these variables, not just temperature, if regeneration rates were to be compared.

Detailed histological observations were made on 40 selected limbs from the experiments in Group B. These limbs were representative of all stages of regeneration from one week to 33 weeks after amputation. All limbs were serially sectioned and reconstructions were made of the older limbs by means of camera lucida drawings of the sections. Sections were 10 μ in thickness and stained with Harris' hematoxylin and eosin. Limbs were fixed in Bouin's fluid.

RESULTS

A. *Continuous treatment*

In this section, experiments using thyroxine or desiccated thyroid continuously from the time treatment was initiated throughout regeneration until termination of the experiment will be described. It was found that weak concentrations of thyroxine ($1 \cdot 10^{-7}$) or desiccated thyroid delayed the onset of proliferation of the blastema slightly, but had little permanent effect on the regenerate. Preliminary experiments with a thyroxine concentration of $1 \cdot 10^{-6}$ indicated that the higher dosage had a greater inhibiting effect. Section B will deal with further experiments using the higher dosage. It is important, however, to consider the mild effects of the low concentration because they are in the same direction as the effects of the higher concentration, which both gives validity to the results and helps to explain the mechanism of action of the drug. Table I gives the details of the experiments. Figure 6 summarizes the results.

Experiments 5(1) and 5(2). Thyroxine $1 \cdot 10^{-6}$ was administered to 21 newts (see materials and methods) throughout the experiment and the left forearms of 17 surviving animals were amputated one month after beginning treatment (experiment 5(1)). Amputated at the same time were the left forearms of two other groups of newts which had been kept under identical laboratory conditions (see materials and methods) without the thyroxine: the first group (5C) consisted of 15 control newts; the second group (5(2), 19 newts) was begun on thyroxine $1 \cdot 10^{-7}$ at the time of amputation. The results were not striking.

There was a mild but definite delay in the onset of growth in the treated cases. Table II expresses this as number of limbs in each stage of regeneration at 3, 4 and 5 weeks after amputation. Treatment with thyroxine prior to amputation (5(1)), did not produce any greater effect than treatment begun at amputation (5(2), Table II), contrary to what might have been expected from certain investigations of the effects of hormones on regeneration (6, 10, 18). When the experiment was terminated 12 weeks after amputation, there was no significant difference in size or differentiation between the control and experimental groups (5C and 5(1)).

Experiments 6(1) and 6(2). The right forearms of the same groups of newts were amputated 5 weeks after the amputation of the left forearms (above). Thyroxine $1 \cdot 10^{-7}$ was continued in the initial group (5(1) newts) to give a group which had been pretreated for slightly over two months pre-amputation continuing on the same dosage of thyroxine (experiment 6(1)). Since this concentration had not affected regeneration very greatly (see above), the thyroxine dosage was raised to $1 \cdot 10^{-6}$ at the time of amputation in the remaining group of experimentals (6(2)). Ten of the controls were amputated (6C). The results are shown in Table II. Beginning thyroxine two months before amputation (6(1)) gave about the same result (slight retardation of initial proliferation) as beginning this dosage one month before amputation (5(1)). On the other hand, the regenerates of the animals receiving the higher concentration (6(2)) grew very little (Table II). Death of the animals terminated this experiment (6(2)) a little over 5 weeks after amputation, but the two regenerates of the animals that survived to that time were greatly stunted.

Experiments 7(1) and 7(2). One week before amputation of the right forearms (7(1)), 15 newts were begun on desiccated thyroid feedings (see materials and methods). Three weeks after the amputation of the right forearms, the left forearms were amputated (7(2)). The right and left forearms of 10 control newts were amputated at the same times (7C(1) and 7C(2)). The results of this treatment were similar to the results with the low concentration ($1 \cdot 10^{-7}$) of thyroxine and consisted of a slight delay in the initial onset of proliferation of the regenerate (Table II). Differences in delay between the two experiments are not significant, since it is difficult to give a constant dosage when feeding thyroid.

Experiments 7z(1) and 7z(2). A second group of experiments was run in conjunction with 7(1) and 7(2) in order to study the higher concentration of thyroxine further. A group of 5 newts were begun on thyroxine $1 \cdot 10^{-6}$ three weeks after amputation of the right forearms (7z(1), controls 7C(1)). At the time thyroxine was begun, the left forearms were amputated (7z(2), controls 7C(2)). The limbs which had been allowed to regenerate for three weeks before therapy was begun (7z(1)) were in the cone, paddle or toebud stage (Table II) when thyroxine was begun and they continued to grow and differentiate at

the same rate as the controls. The survivors were slightly smaller than the controls. On the other hand, the limbs which had been treated with this high concentration of thyroxine from the time of amputation (7z(2)) showed no growth during the course of the experiment (terminated 4 weeks after amputation by death of the animals).

B. *Discontinuous treatment*

Since it was desirable to explore more fully the effects of the higher concentration of thyroxine, additional experiments were done in which the drug was administered for a shorter period of time. This approach, which permitted the animals to be followed longer, was very revealing. Most important was the fact that regenerates treated during the stage of proliferation and differentiation were not able to recuperate from the effects of treatment (experiments 8(1) and 4(2)).

Experiments 8(1), 8(2) and 8(3). Nineteen newts were begun on thyroxine $1 \cdot 10^{-6}$ 9 days before amputation of the right forelimb (experiment 8(1)). Four weeks after amputation, the concentration of thyroxine was gradually lowered

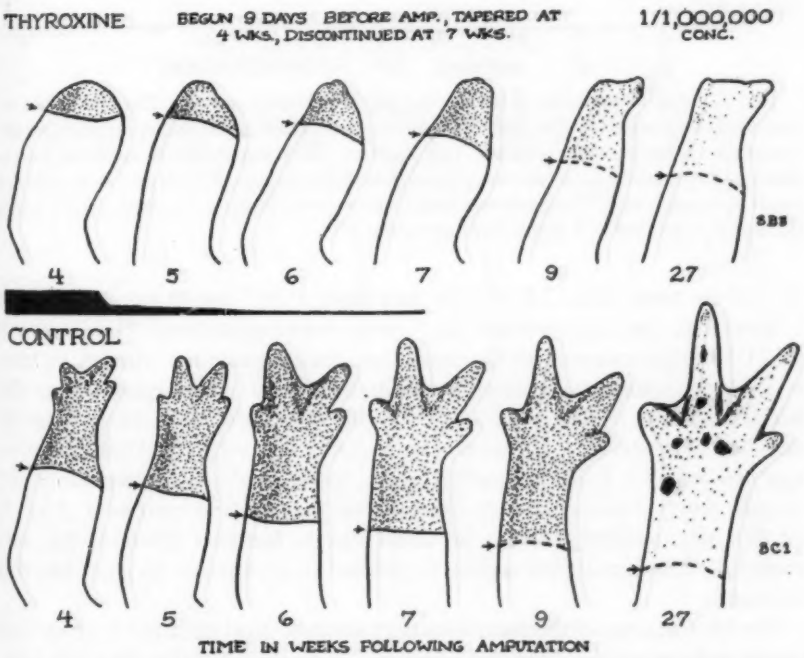


FIG. 1. Camera lucida drawings of regenerating limbs. The solid black line represents duration of treatment. Arrow indicates amputation level. Experiment 8(1).

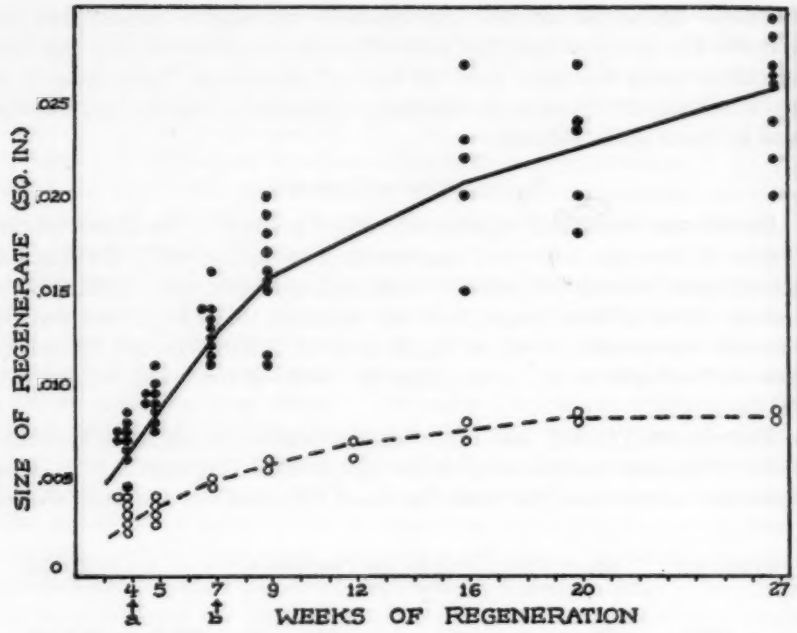


FIG. 2. Effect of thyroxine $1 \cdot 10^{-6}$ on the size of regenerating limbs. The size of the regenerate is represented by the area of its camera lucida drawing (as square inches of an unmagnified drawing) on the ordinate. Time in weeks after amputation is indicated on the abscissa. Each regenerate in the treated group is shown as a clear circle and in the control group by a solid circle. Thyroxine was begun 9 days before amputation, tapered at 4 weeks (a), and discontinued at 7 weeks (b). Experiment 8(1).

($5 \cdot 10^{-7}$ for three days, $2.5 \cdot 10^{-7}$ for two days, $1 \cdot 10^{-7}$ for 10 days, $5 \cdot 10^{-8}$ for 7 days) and then discontinued (at 7 weeks post-amputation). Nine controls (8C(1)) were amputated at the same time (right forelimbs). As can be seen in Table II, treatment produced a considerable delay in the appearance of the initial stages of regeneration. Figure 1 illustrates this effect (camera lucida drawings of a typical experimental case and a typical control). After thyroxine was tapered at 4 weeks post-amputation, the treated regenerates continued to grow slowly, but they did not recover from the effects of treatment (Figs. 2, 19, 20). More striking than the size effect was the fact that differentiation was abnormal. The histological section is devoted to an attempt to elucidate this finding.

The left forearms of the two newts that survived treatment (8A5, 8B8) were amputated two weeks after thyroxine was discontinued (8(2)). The left forearms of the nine original controls were amputated at the same time. The con-

trol and experimental rates of regeneration were essentially identical (Fig. 3) and the resulting regenerates normal, although slightly smaller 23 weeks after amputation, when the experiment was terminated. Thirty-two weeks after the initial amputation (8(1)) of the right forelimbs of these animals, by which time growth had ceased and ossification begun in the regenerates, the right forelimbs of 8A5 and 8B8 were reamputated through the abnormal regenerates (one mm. distal to the previous level of amputation). The reamputated controls and experimentals regenerated at the same rate, differentiated normally and were similar in size (experiment 8(3)). The previous treatment had not affected the ability of the cells to grow after a new amputation.

Experiments 4(1) and 4(2). It is possible to explore the effect of thyroxine further by limiting the dosage to particular periods in regeneration. In experiment 4(1), 16 newts were begun on thyroxine $1 \cdot 10^{-6}$ at the time of amputation of the right forelimb and two weeks later thyroxine was discontinued while the regenerates were still in the early blastema stage (temperature 20–23°C.). Sixteen controls (4C) had been amputated at the same time. The experimentals showed a delay in the onset of growth (Table II), but no permanent effect was

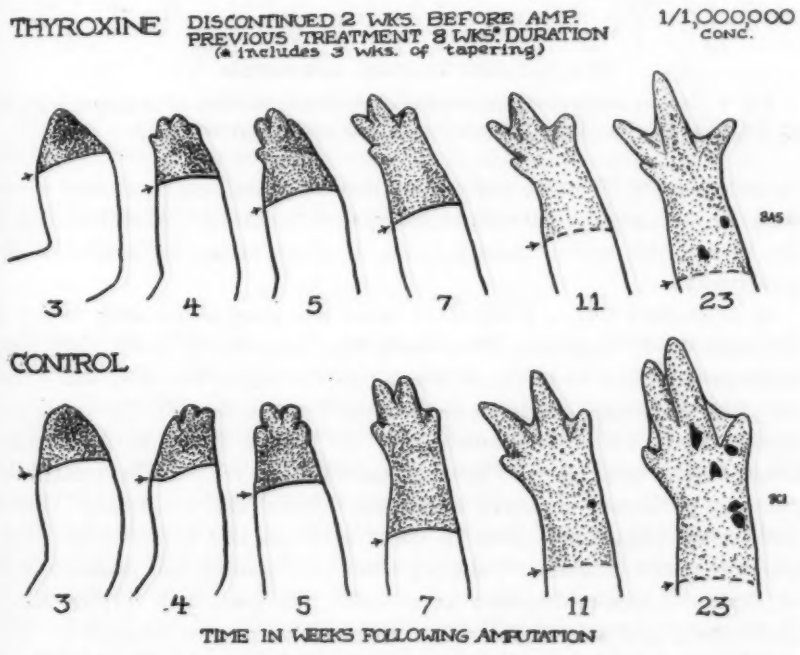


FIG. 3. Camera lucida drawings of regenerating limbs. Treatment was discontinued before amputation. Arrow indicates amputation level. Experiment 8(2).

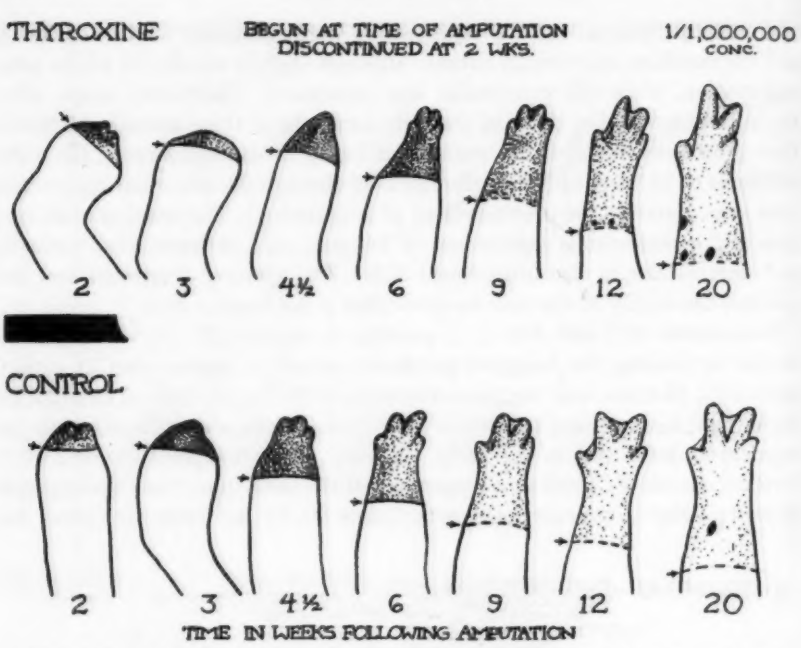


FIG. 4. Camera lucida drawings of regenerating limbs. Duration of treatment is shown by the solid black line. Arrow indicates amputation level. Experiment 4(1).

produced and at 20 weeks post-amputation the experimentals showed essentially the same growth and differentiation as the controls. Figure 4 illustrates the results with typical camera lucida drawings during the course of the experiment.

In experiment 4(2), a group of 16 newts was given a two week course of thyroxine $1 \cdot 10^{-6}$ beginning three weeks after amputation of the right forelimbs (temperature 18–19°C.), at which time the regenerates were still in the early blastema stage, but about to begin proliferation (Fig. 8). Thyroxine was tapered 5 weeks after amputation (to $5 \cdot 10^{-7}$ then $2 \cdot 10^{-7}$) and discontinued 6 weeks after amputation. Figure 5 illustrates the results. The treated regenerates proliferated unusually slowly and differentiated abnormally. Fifteen weeks after amputation, three of the 6 survivors had permanently small, paddle-shaped regenerates. The other three were stunted, but possessed toes which showed various degrees of abnormality. There was little further change at 23 weeks post-amputation, when the experiment was terminated. This experiment resembles experiment 8(1), with which it has in common the fact

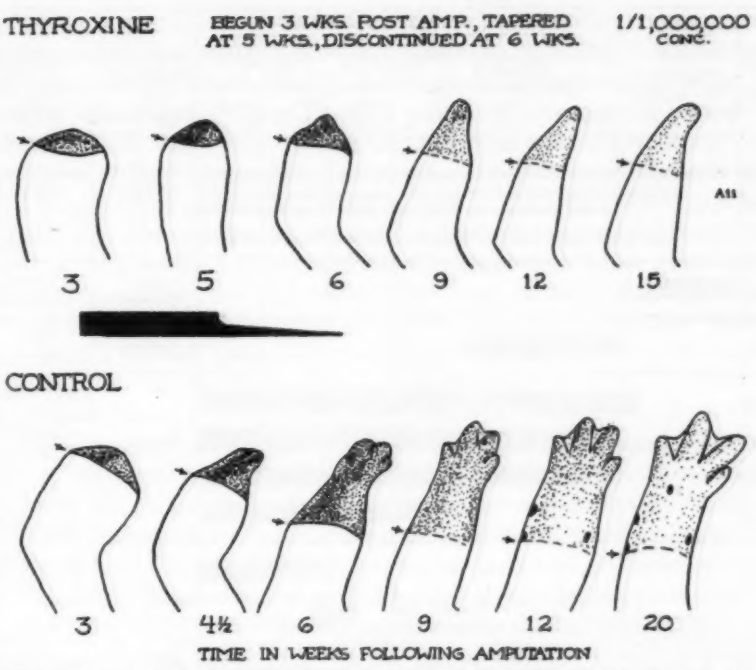


FIG. 5. Camera lucida drawings of regenerating limbs. Duration of treatment is shown by the solid black line. Arrow indicates amputation level. Experiment 4(2).

that the stage of proliferation and differentiation was treated. Figure 6 summarizes the results.

C. *Histological observations*

Regenerating limbs one to six weeks after amputation (*Thyroxine* $1 \cdot 10^{-6}$). Between one and two weeks after amputation (Fig. 7) the thyroxine treated regenerating limbs, like the controls, accumulate blastema cells by apparent dedifferentiation of the old tissues. Epithelium piles up over the cut ends of the bones in mounds 8–10 cells deep. Muscle and other inner tissues go through the usual steps of dissolution and transformation.

By three weeks of age, proliferation was well underway in all of the controls in experiment 8(1), many of the regenerates having formed toes. Half of the 18 treated regenerates had not begun to grow and were still in the early blastema stage (Table II). Histologically, this retarded blastema is not strikingly abnormal (Figs. 9, 10), its main feature being that it has not begun to pro-

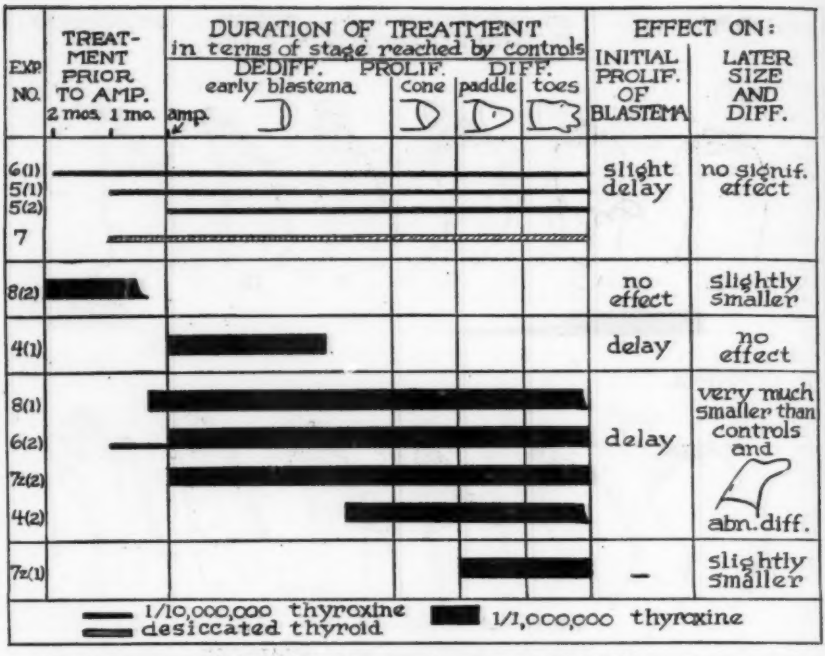


FIG. 6. Summary of the results of the experiments reported in this paper. The amounts and durations of treatments are indicated by the horizontal bars. Abbreviations: abn.—abnormal; amp.—amputation; dediff.—dedifferentiation; diff.—differentiation; prolif.—proliferation; signif.—significant.

iferate appreciably. The cartilage differentiation around the ends of the cut bones (Figs. 9, 10) is normal in amount for this stage.

By 4 weeks of age, all of the controls in experiment 8(1) had formed toes, whereas most of the thyroxine treated regenerates were in the paddle stage (Table II). Proliferation, though retarded, has occurred (Fig. 11). Cartilage differentiation, however, is more advanced in the thyroxine treated "paddle" (Fig. 11) than is usual for the paddle stage (Fig. 14) of regeneration. The amount of cartilage in the treated regenerate is not, however, unusual for this age, as all of the controls had formed toes and would be at least as advanced as the untreated regenerate shown in Figure 13. The difference would seem to be that by the time a control has differentiated this much cartilage the distal end is growing rapidly and digits have appeared. Indeed, it can be seen by comparing Figures 11 and 14 that the untreated paddle has laid down a larger distal mass (toe region) in proportion to the rest of the regenerate than has the treated paddle, yet the treated regenerate, in accordance with its age and not prematurely, has already differentiated cartilage in the carpal region.

A little later, the abnormalities in the cartilage pattern become more apparent (experiments 4(2), 8(1)). In normal regeneration, the carpals differentiate out of a pre-cartilage mass (Fig. 14) as distinct entities separated by layers of condensed cells (Fig. 13) which later differentiate into connective tissue membranes (Figs. 17, 21). There is, however, a tendency in thyroxine treated regenerates for these condensed cells between the carpals to become cartilage (Fig. 12), which may explain the "fused" pattern seen in some older regenerates (Figs. 18, 22). The cartilage of the distal region differentiates, although the number of cells here is deficient, and abnormalities result in the metacarpals and phalanges (to be described below). The other tissues in the regenerates 4–6 weeks of age do not present as definite a picture of abnormality. Muscle differentiation has not progressed very far at this stage. The epithelium is thinner than normal due to the continual molting.

Regenerating limbs 17 to 33 weeks after amputation (*Thyroxine* $1 \cdot 10^{-6}$). The regenerates treated during the phase of proliferation and differentiation (Figs. 1, 5) did not recover from the effects of treatment described above. Figures 15 and 16 show two of the regenerates in experiment 4(2) which remained paddle-shaped and Figure 17 shows a control from the same experiment at a similar age. The abnormal regenerates are composed primarily of cartilage, which has differentiated in an abnormal pattern. The carpals tend to be long and abnormally shaped and are deficient in number. Distal elements are deficient and limited to one or two abnormal metacarpals in the limbs which remained paddle-shaped. Muscle is deficient. One of the treated limbs which developed 4 toes (stunted) is shown in Figure 18. Three of the metacarpals are fused together, the phalanges are incomplete, and the carpals are deficient and abnormally shaped.

Figures 19 and 20 show the external growth of the limbs illustrated in Figures 21, 22 and 23 (experiment 8(1)). Thyroxine was discontinued at about the time of the first photographs (Fig. 19), at which time abnormal differentiation was apparent in the treated cases. After thyroxine was discontinued, some growth occurred (Fig. 20) but the effect was essentially permanent. Histological sections of these regenerates 32 weeks after amputation (Figs. 22, 23) reveal abnormally shaped carpals deficient in number, sometimes fused and generally so unusual as hardly to deserve the name carpals. There are two abnormal cartilages approximating metacarpals and a small "phalanx" in each of these limbs. Ossification has begun in the controls of this age (Fig. 21) and is beginning in the treated regenerates (Figs. 22, 23). Muscle is deficient in the treated regenerates. The epithelium is essentially normal. (Figs. 21, 22 and 23 are magnified 25 X, whereas the preceding photomicrographs are magnified 50 ×.)

The main features of the histological abnormalities resulting from thyroxine (1.10^{-6}) treatment during the period of proliferation and differentiation can

be summarized as follows: (a) the pattern of cartilage differentiation is abnormal; (b) the amount of cartilage present seems excessive relative to other tissues formed, but the total cartilage mass is not any greater than that in the controls and cartilage does not form prematurely; (c) there is a tendency toward local overgrowth of cartilage, as evidenced by fusion of carpals and other elements.

DISCUSSION

The use of different concentrations of thyroxine in different periods of regeneration permits certain tentative conclusions about the action of thyroxine in regeneration to be drawn. In the first place, treatment with thyroxine ($1 \cdot 10^{-6}$, $1 \cdot 10^{-7}$) prior to amputation of the limb did not significantly inhibit or contribute to inhibition of adult *Triturus* limb regeneration, contrary to the situation in the frog tadpole, where thyroid therapy in weak dosage given prior to amputation completely inhibits limb regeneration (6, 10). Thyroidectomy and thiourea have various effects on regeneration in the frog tadpole (19–21), but thiourea does not restore regenerative capacity once lost (21). In the salamander, thyroidectomy causes retarded or abnormal regeneration (22, 23). It seems unlikely that thyroid hormone itself is directly involved in the loss of regenerative capacities by higher animals. The changes produced by thyroid in the frog would seem to be specific for the frog.

Regarding the effects of thyroxine given during regeneration of the limb (Fig. 6), several comments can be made. Of importance in interpreting these results is the fact that the doses of thyroxine used were toxic to the newts. The death rate (Table I) and poor condition of the newts cannot be correlated with the results in these experiments (Fig. 6), nor would non-specific toxicity be expected to inhibit regeneration (24). It seems likely, however, that thyroxine in these high doses might have had a direct toxic effect on cellular metabolism in such a way as perhaps to interfere with mitosis or other activity of the regenerating cells. In this regard, it is interesting that a number of studies of the effect of hyperthyroidism on cellular division report a depressing effect (25–30). In the present experiments, thyroxine delayed and/or partially inhibited proliferation of the blastema.

In addition to the delay in growth, thyroxine ($1 \cdot 10^{-6}$), if given during the stage of proliferation and differentiation, produced abnormal differentiation and stunting that were permanent even after treatment was discontinued. Abnormal, relatively excessive cartilage characterized such regenerates, but cartilage differentiation did not appear to be accelerated by the treatment (see section on histological studies) and, moreover, there was no evidence that thyroxine administered late in regeneration, prior to regeneration, or during only the first two weeks of regeneration had any effect on cartilage. These facts suggest that the abnormal cartilage differentiation might have been a secondary

effect of thyroxine given in this particular stage (early growth and differentiation) of regeneration. Indeed, histological observations (see section C) suggested that the balance between proliferation and differentiation was disrupted by treatment. At the time differentiation began (a time which was similar in controls and experimentals), the treated regenerates had proliferated less than the controls and were deficient in cellular numbers. The importance of a critical mass or number of cells to normal differentiation in limb development has been pointed out by previous authors (31–33). It seems possible that in the present case the abnormalities resulted because an insufficient mass of cells was produced for correct differentiation of the cartilage pattern. The fact that the whole pattern of the regenerate is laid down in a narrow range of time after proliferation first begins would explain the permanency of the abnormalities.

The idea that a disturbance in the balance between proliferation and differentiation might cause regenerative abnormalities is not a new one. The histological picture here strikingly resembles that of the best limb regenerates artificially induced in frogs (34, 35) and Singer (36, p. 457) suggests, in relation to nerve-induced heteromorphic limb regenerates in frogs, that "If by some means the growth potential of the induced regenerate could be enhanced further, or if the tendency toward early histogenesis be diminished, a regenerate of proportions and structure more like that of the normal limb may be obtained." Fusion of carpals and other skeletal abnormalities have been reported in salamander limb regenerates disturbed by a variety of causes (37–40), which might have in common some interference with the balance between proliferation and differentiation.

It is conceivable (see discussions, 41, 42) that the formation of a thick connective tissue pad over the end of an amputated limb is a non-specific picture of non-regeneration. In this vein, we might suggest that the abnormal pattern of cartilage differentiation seen here is a non-specific picture of regeneration that got started, as it were, but lacked the necessary growth potential to reproduce the normal limb pattern. Indeed, workers who have tested a range of concentrations of beryllium, an agent capable of completely suppressing regeneration, have reported this second effect (heteromorphic regeneration) at lower concentrations (41, 43, 44). Moreover, it is possible to produce either complete suppression or heteromorphic growths by denervating the early blastema (45, 46). It could even be said that the untreated frog fits into the first category, no regeneration normally occurring, and that the stimulated frog limb (34–36) has been raised to the level of the second category, heteromorphic regeneration. If this is a general rule, then one might expect to get the picture of non-regeneration with higher concentrations of thyroxine, if it is possible to inhibit mitosis completely with thyroxine.

The results of the present experiment resemble those of denervation (45)

and partial denervation (46) and it is possible (as has been suggested for colchicine, 47) that the action of thyroxine is via the nervous system. The fact, however, that denervation represses mitosis (17) would make it unnecessary to explain the similarity of these results by anything besides an effect on cell division. Although it is not in the scope of this discussion to review the literature on the action of inhibiting agents on limb regeneration in salamander larvae, it is interesting that both colchicine (48) and x-rays (49, 50), agents capable of inhibiting cellular division, produce the same picture of regression as is produced by denervation (51).

The final observation (Fig. 6), that it does not seem to be necessary to begin treatment of the regenerating adult *Triturus* limb until after amputation and wound healing are completed in order to produce abnormalities, has been reported for denervation (45) and seems to be true for beryllium (44). As a matter of fact, it is not necessary to denervate the regenerating limb earlier than 10–13 days after amputation (25°C.) to get complete suppression of the process and this period, which corresponds to the first peak in the mitotic rate of the blastema, has been called a "period of lability" by Singer and Craven (45, p. 303). Hypophysectomy, which suppresses regeneration partially or completely depending on the time performed, was thought to act on the wound healing process which lasts 5–6 days after amputation (42, 52), but recently it has been shown that replacement therapy is most effective when administered 15 days after amputation and least effective at the time of amputation (53). These facts would seem to point to the stage of initial proliferation of the blastema (10–20 days post-amputation) as a most important phase.

In conclusion, the idea could be suggested that many of the inhibiting agents discussed above might have in common the ability to depress cellular proliferation. This one idea could tie together most of the results discussed. One could speculate that marked inability of the cells in a limb to proliferate might be followed by scar formation or even regression, whereas moderate but insufficient proliferation might fail to provide the volume of cells required for that delicate stage of differentiating the normal limb pattern.

SUMMARY

The present study of the effect of hyperthyroidism on limb regeneration in the adult newt, *Triturus viridescens*, stems from an interest in factors which cause animals to lose this remarkable regenerative capacity. Thyroid hormone brings about both metamorphosis and loss of the power of limb regeneration in the frog. Unlike the case in the frog, treatment with thyroxine prior to limb amputation did not prevent limb regeneration in the newt. It was found, however, that thyroxine given *during* regeneration partially inhibited proliferation of the regenerate. Moreover, thyroxine ($1 \cdot 10^{-6}$) administered through the stage of proliferation and differentiation of the regenerates produced abnor-

mally differentiated, stunted regenerates which did not recover after treatment was discontinued. Yet, treatment begun after the limb had begun to grow and differentiate or discontinued before this stage did not upset differentiation. These combined studies, together with histological studies, suggest that the abnormal differentiation might have been a secondary effect of thyroxine given in this particular stage (early proliferation and differentiation) of regeneration. Indeed, the primary difficulty seemed to be that the regenerate lacked the normal quota of cells at the time of differentiation of the limb pattern. The effects of thyroxine are discussed in relation to other types of inhibition of limb regeneration in salamanders and it is concluded that these effects are not specific and may even be related in basic mechanism (inhibition of cell division).

LITERATURE CITED

1. Lynn, W. G. and H. E. Wachowski: The thyroid gland and its functions in cold-blooded vertebrates. Quart. Rev. Biol., 1951, **26**: 123–168.
2. Gudernatsch, J. F.: Feeding experiments on tadpoles. I. The influence of specific organs given as food on growth and differentiation. Arch. Entwmech., 1913, **35**: 457–483.
3. Gudernatsch, J. F.: Feeding experiments on tadpoles. II. A further contribution to the knowledge of organs with internal secretion. Am. J. Anat., 1914, **15**: 431–482.
4. Romeis, B.: Der Einfluss verschiedenartiger Ernährung auf die Regeneration bei Kaulquappen (*Rana esculenta*). Arch. Entwmech., 1913, **37**: 183–216.
5. Romeis, B.: Experimentelle Untersuchungen über die Wirkung innersekretorischer Organe. II. Der Einfluss von Thyreoidea- und Thymusfütterung auf das Wachstum, die Entwicklung und die Regeneration von Anurenlarven. Arch. Entwmech., 1914, **40**: 571–652.
6. Speidel, C. C.: Studies of hyperthyroidism. VI. Regenerative phenomena in thyroid-treated amphibian larvae. Am. J. Anat., 1929, **43**: 103–165.
7. Adams, A. E.: Anterior-pituitary extract (phyone) and regeneration of the hind leg in *Triturus viridescens*. Anat. Rec., 1932, *52*, Suppl., **1**: 45–46.
8. Herrell, W. E.: Growth and regeneration of tissue in frog tadpoles following the administration of an extract of the anterior pituitary gland. Anat. Rec., 1934, **59**: 47–68.
9. Puckett, W. O.: The effects of intra-peritoneal injections of pituitary substances on the rate of tail regeneration in frog tadpoles. Anat. Rec., 1938, **71**: 337–348.
10. Warren, A. E. and C. M. Bower: The influence of normal and induced metamorphosis on hind limb regeneration in *Rana sylvatica*. Anat. Rec., 1939, **73**: suppl., **2**: 55–56.
11. Pawlowsky, E. N.: Hyperthyroidismus und Regeneration. Arch. Mikr. Anat. Entwmech., 1923, **99**: 620–627.
12. Richardson, D.: Thyroid and pituitary hormones in relation to regeneration. II. Regeneration of the hind leg of the newt, *Triturus viridescens*, with different combinations of thyroid and pituitary hormones. J. Exper. Zool., 1945, **100**: 417–427.
13. Kirkwood, E. S.: The regeneration of the anterior limb of *Triturus viridescens* under normal and varying thyroid conditions. Thesis, 1931, Mount Holyoke College.
14. Belkin, R.: Régénération des extrémités de l'axolotl pendant la métamorphose. Compt. rend. Soc. biol. Paris, France, 1934, **115**: 1162–1163.
15. Adams, A. E., A. Kudor and L. Richards: The endocrine glands and molting in *Triturus viridescens*. J. Exper. Zool., 1932, **63**: 1–56.

16. TAYLOR, A.: The effect of athyroidism and hyperthyroidism on the oxygen consumption of the adult salamander. J. Exper. Zool., 1939, **81**: 135–146.
17. SINGER, M.: The influence of the nerve in regeneration of the amphibian extremity. Quart. Rev. Biol., 1952, **27**: 169–200.
18. GROBSTEIN, C.: The role of androgen in declining regenerative capacity during morphogenesis of the *Platypoecilus maculatus* gonopodium. J. Exper. Zool., 1947, **106**: 313–344.
19. ALLEN, B. M.: The relation of the thyroid gland to regeneration in *Rana pipiens*. Anat. Rec., 1918, **14**: 85–86.
20. GHIDONI, M. B.: Effects of thyroid inhibitors upon tail regeneration in the tadpole. Growth, 1948, **12**: 181–202.
21. PAEDON, A. M.: The effects of thiourea on limb regeneration in the tadpole. Growth 1953, **17**: 21–44.
22. WALTER, F. K.: Schildrüse und Regeneration. Arch. Entwmech., 1910, **31**: 91–130.
23. RICHARDSON, D.: Thyroid and pituitary hormones in relation to regeneration. I. The effect of anterior pituitary hormone on regeneration of the hind leg in normal and thyroidectomized newts. J. Exper. Zool., 1940, **83**: 407–430.
24. KARCZMAR, A. G. and G. G. BERG: Effects of dibenzanthracene and methylcholanthrene on forelimb regeneration of urodele larvae. J. Morphol., 1952, **91**: 479–514.
25. WOODRUFF, L. L. AND W. W. SWINGLE: The effects of thyroid and some other endocrine products on paramecium. Am. J. Physiol., 1924, **69**: 21–34.
26. TORREY, H. B., M. C. RIDDLE AND J. L. BRODIE: Thyroxin as a depressant of the division rate of paramecium. J. Gen. Physiol., 1925, **7**: 449–460.
27. TORREY, H. B.: Thyroxin as a depressant of cell division; its effect on the cleavage and early development of sea urchin and ascidian. Endocrinology, 1928, **12**: 65–80.
28. BUTLER, E. G.: The thyroid and the rate of cell division. Proc. Soc. Exper. Biol. & Med., 1928, **26**: 231–233.
29. LATTA, J. S. AND J. Z. DAVIS: The effects of thyroxin in varying concentrations on the growth of fibroblasts in vitro. Arch. Exper. Zellforsch., 1938, **21**: 427–435.
30. SMELSER, G. K. AND V. OZANICS: Effect of thyroid hormone on mitosis and regeneration in corneal epithelium. J. Cell. & Comp. Physiol., 1954, **43**: 107–117.
31. GUYÉNOT, E. AND O. SCHOTTÉ: Relation entre la masse du bourgeon de régénération et la morphologie du régénérat. Compt. rend. Soc. biol. Paris, France, 1923, **89**: 491–493.
32. DANFORTH, C. H.: Morphology of the feet in polydactyl cats. Am. J. Anat., 1947, **80**: 143–171.
33. BRETSCHER, A. AND P. TSCHUMI: Gestufte Reduktion von Chemisch behandelten *Xenopus*-Beinen. Rev. Suisse Zool., 1951, **58**: 391–398.
34. ROSE, S. M.: Methods of initiating limb regeneration in adult Anura. J. Exper. Zool., 1944, **95**: 149–170.
35. GIDGE, N. M. AND S. M. ROSE: The role of larval skin in promoting limb regeneration in adult anura. J. Exper. Zool., 1944, **97**: 71–93.
36. SINGER, M.: Induction of regeneration of the forelimb of the postmetamorphic frog by augmentation of the nerve supply. J. Exper. Zool., 1954, **126**: 419–472.
37. SCHMALHAUSEN, J.: Über die Beeinflussung der Morphogenese der Extremitäten vom Axolotl durch verschiedene Faktoren. Arch. Entwmech., 1925, **105**: 483–500.
38. BRUNST, V.: Zur Frage nach dem Einfluss des Nervensystems auf die Regeneration. Arch. Entwmech., 1927, **109**: 41–53.

39. BRUNST, V.: Über die Bedeutung der Funktion für die definitive Skeletbildung der Extremitäten bei der Regeneration. Arch. Entwmech., 1932, **125:** 640–662.
40. DENT, J. N.: A study of regenerates emanating from limb transplants with reversed proximodistal polarity in the adult newt. Anat. Rec., 1954, **118:** 841–856.
41. THORNTON, C. S.: Beryllium inhibition of regeneration. III. Histological effects of beryllium on the amputated forelimbs of *Amblystoma* larvae. J. Exper. Zool., 1951, **118:** 467–494.
42. SCHOTTÉ, O. E. AND A. B. HALL: Effects of hypophysectomy upon phases of regeneration in progress (*Triturus viridescens*). J. Exper. Zool., 1952, **121:** 521–560.
43. THORNTON, C. S.: Beryllium inhibition of regeneration. I. Morphological effects of beryllium on amputated fore limbs of larval *Amblystoma*. J. Exper. Zool., 1949, **84:** 459–494.
44. SCHEUING, M. AND R. SIDMAN: The influence of small quantities of beryllium nitrate infused into the early regenerate of the forelimb of the adult newt, *Triturus*. Anat. Rec., 1953, **117:** 578.
45. SINGER, M. AND L. CRAVEN: The growth and morphogenesis of the regenerating fore limb of adult *Triturus* following denervation at various stages of development. J. Exper. Zool., 1948, **108:** 279–308.
46. SINGER, M. AND F. R. L. EGLOFF: The nervous system and regeneration of the forelimb of adult *Triturus*. VIII. The effect of limited nerve quantities on regeneration. J. Exper. Zool., 1949, **111:** 295–314.
47. FLINKER, D. AND R. SIDMAN: The influence of infusions of colchicine on the nerve and the early regenerate of the forelimb of the adult newt, *Triturus*. Anat. Rec., 1953, **117:** 577.
48. THORNTON, C. S.: The effect of colchicine on limb regeneration in larval *Amblystoma*. J. Exper. Zool., 1943, **92:** 281–296.
49. BUTLER, E. G.: The effects of x-radiation on the regeneration of the forelimb of *Amblystoma* larvae. J. Exper. Zool., 1933, **65:** 271–316.
50. BUTLER, E. G. AND W. O. PUCKETT: Studies on cellular interaction during limb regeneration in *Amblystoma*. J. Exper. Zool., 1940, **84:** 223–240.
51. BUTLER, E. G. AND O. E. SCHOTTÉ: Histological alterations in denervated non-regenerating limbs of urodele larvae. J. Exper. Zool., 1941, **88:** 307–342.
52. HALL, A. B. AND O. E. SCHOTTÉ: Effects of hypophysectomies upon the initiation of regenerative processes in the limb of *Triturus viridescens*. J. Exper. Zool., **118:** 363–388.
53. SCHOTTÉ, O. E. AND D. A. B. LINDBERG: Effect of xenoplastic adrenal transplants upon limb regeneration in normal and hypophysectomized newts (*Triturus viridescens*). Proc. Soc. Exper. Biol. & Med., 1954, **87:** 26–29.

PLATE 1

Explanation of Figures

Figures are unretouched photomicrographs. Magnification ×50.

Fig. 7. Regenerating limb 8 days after amputation treated with thyroxine from time of amputation (B9, experiment 4(1)).

Fig. 8. Regenerating limb (A1) in early blastema stage fixed at time (3 weeks) thyroxine was begun in experiment 4(2).

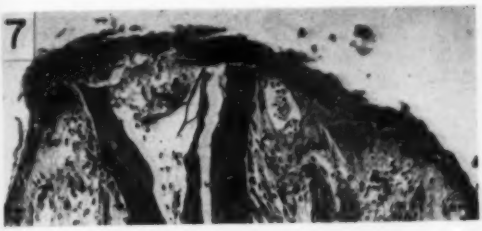
Fig. 9. Retarded regenerating limb 19 days after amputation treated with thyroxine from time of amputation (8B2, experiment 8(1)).

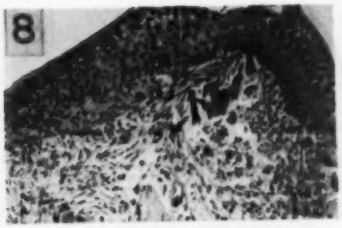
Fig. 10. Retarded regenerating limb 21 days after amputation treated with thyroxine from time of amputation (8B3, experiment 8(1)).

Fig. 11. Retarded regenerating limb 31 days after amputation treated with thyroxine from time of amputation (8B9, experiment 8(1)). In the lower part of the picture, the ulna is on the left, radius on the right.

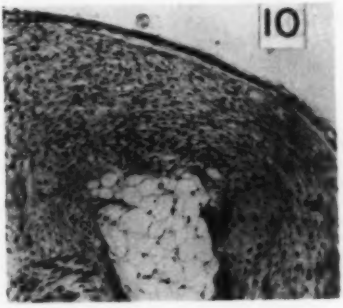
Fig. 12. Retarded regenerating limb 34 days after amputation treated with thyroxine from time of amputation (8A8, experiment 8(1)). The carpal region and two of the abnormal metacarpals are shown (the limb had 4 small toes). The cartilage in the lower left-hand corner is the end of the radius.

Fig. 13. Control regenerating limb 27 days after amputation in early stages of differentiation. (IIIC1, experiment 5). The ulna and radius, not shown, are to the left and right, respectively.

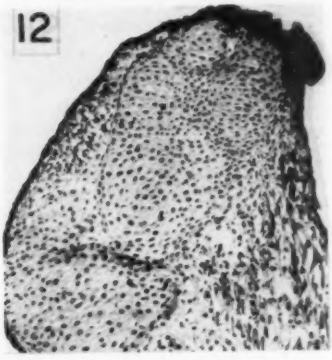

7


8

9


10

11


12

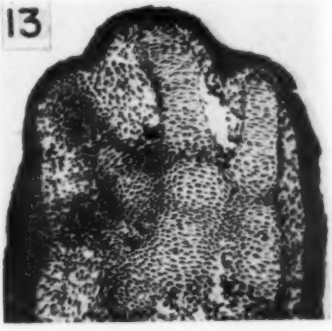

13

PLATE II

Explanation of Figures

Figures are unretouched photomicrographs. Magnification ×50.

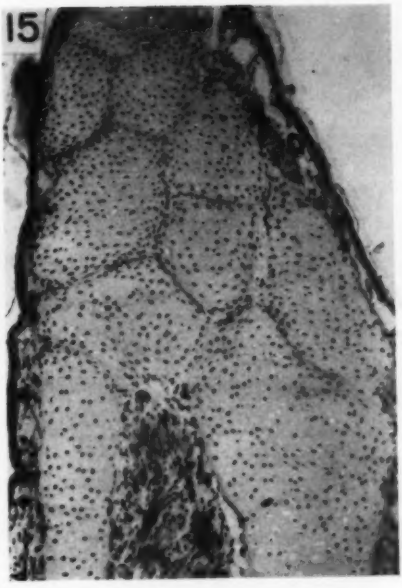
Fig. 14. Control regenerate 21 days after amputation in the paddle stage (8C8, experiment 8(2)). The ulna is to the left and the radius to the right in the lower part of the picture.

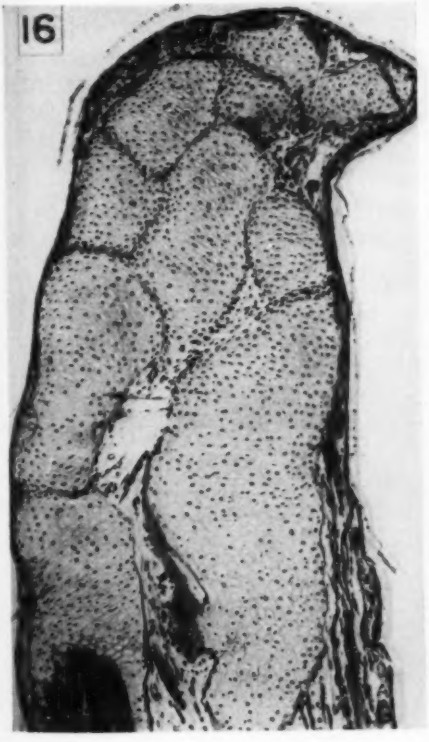
Fig. 15. Regenerating limb approximately 24 weeks (166 days) after amputation treated with thyroxine begun in the stage shown in Figure 8 (IA4, experiment 4(2)). The ulna is to the left and the radius to the right in the lower part of the picture. This limb was paddle-shaped when fixed.

Fig. 16. Regenerating limb approximately 17 weeks (122 days) after amputation treated with thyroxine begun in the stage shown in Figure 8 (A11, experiment 4(2)). The ulna is to the left and the radius to the right. This limb was paddle-shaped when fixed.

Fig. 17. Control regenerate approximately 22 weeks (151 days) after amputation (IIIB4, experiment 4). The ulna is on the left and the radius on the right. Only a part of this well-developed normal regenerate is shown in the section.

14


15


16

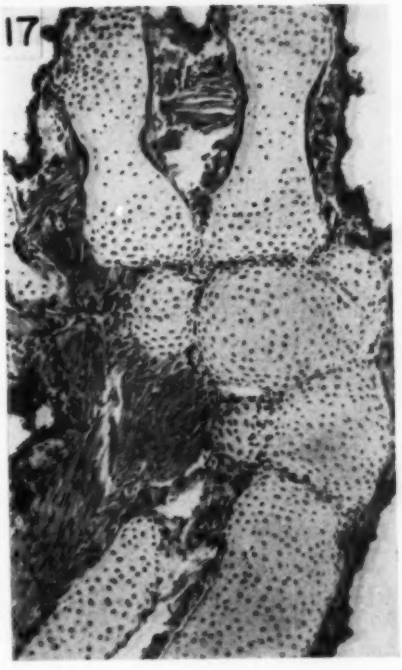

17

PLATE III

Explanation of Figures

Figures are unretouched photographs or photomicrographs at the magnifications indicated.

Fig. 18. Regenerating limb 28 weeks after amputation treated with thyroxine begun in the stage shown in Figure 8 (IA3, experiment 4(2)). The ulna and radius, not shown, would be on the left and right, respectively. The first metacarpal is separate, but M2, M3 and M4 (only part of which is shown) are fused. ×50.

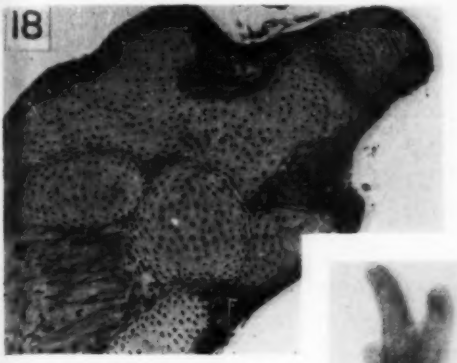
Fig. 19. Regenerating limbs in experiment 8(1) 6 weeks after amputation. (a) 8A5 and (b) 8B8 were treated from 9 days pre-amputation to 7 weeks post-amputation with thyroxine (tapered at 4 weeks). (c) is a typical control (8C1) from the same experiment. Arrows indicate the level of amputation. ×8.

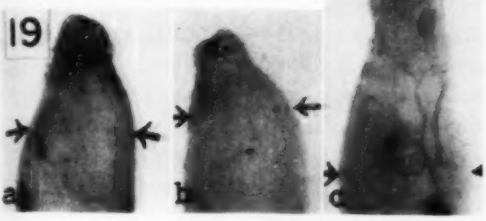
Fig. 20. The regenerating limbs shown in Figure 19 at 23 weeks post-amputation. ×8.

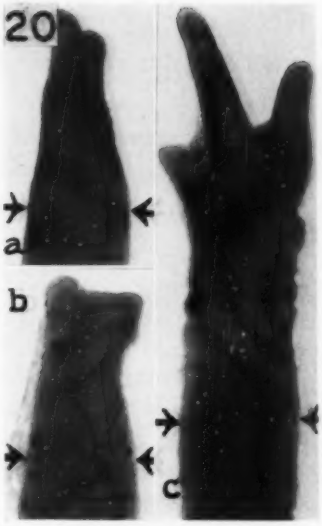
Fig. 21. The regenerating control limb (8C1) shown in Figures 19(c) and 20(c) after fixation at 32 weeks post-amputation. The radius and ulna, not shown, would be on the left and right, respectively. The carpals which appear in this section are the ulnare-intermedium in the lower right corner, parts of the centrale, C1 and C2 (the radiale, C3 and C4 not shown). ×25.

Fig. 22. The treated regenerating limb (8A5) shown in Figures 19(a) and 20(a) after fixation at 32 weeks post-amputation. Note the large distal carpal with which the two small metacarpals articulate. ×25.

Fig. 23. The treated regenerating limb (8B8) shown in Figures 19(b) and 20(b) after fixation at 32 weeks post-amputation. The two metacarpals and 4 of the 5 abnormal carpals that formed are shown. ×25.

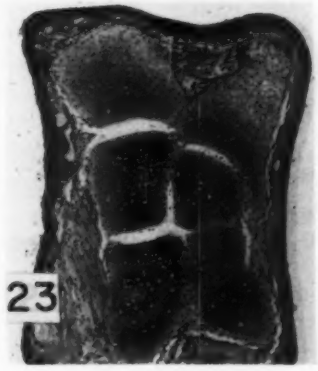

18


19
a
b
c


20
a
b
c

21

22


23

SOME PHYSICO-CHEMICAL ASPECTS OF CALCIFICATION IN VITRO

WILLIAM C. THOMAS, JR.,[1] THOMAS B. CONNOR,[2] AND JOHN EAGER HOWARD

The Department of Medicine, The Johns Hopkins University and Hospital

Received for publication May 31, 1956

The induction *in vitro* of calcification in rachitic rat cartilage matrix by bathing cartilage slices in artificial media was demonstrated by Robison in 1923 (1). By use of similar techniques much information has accrued relative to the experimental requirements necessary for rachitic cartilage to calcify outside the confines of the living organism. It has been found that, for mineral apatite deposition to occur *in vitro*, various tissue enzymes must be present (2) (3) (4) (5). There must also be an adequate concentration of calcium and phosphorus in the bathing solution, else calcification will not occur (3) (6) (7).

Oral feeding of salts of citric acid will induce healing of naturally occurring rickets in infants (8) (9) (10) (11) and of the rickets produced in rats by feeding of low phosphorus diets (12) (13). This curative effect occurs in rachitic infants despite persistence of a low serum phosphorus and an actual decrease in serum calcium concentration, these determinations being made at intervals of several days after institution of citrate therapy (8) (11). In a recent communication (14) it was reported that within three to six hours following the dietary administration of citrates to rats made rachitic by low phosphorus diets, there was a sharp rise in the serum phosphorus concentration. Furthermore, calcification of cartilage slices from these rats, sacrificed a few hours after citrate administration, occurred *in vitro* at lower calcium concentrations in the incubating solution than were required for control rachitic cartilage slices. In addition, cartilage slices from rachitic rats given neutral phosphates intraperitoneally shortly before sacrifice also calcified *in vitro* at lower calcium concentrations than were required for slices from rachitic rats not receiving intraperitoneal phosphorus. *This increased "calcifiability" of the cartilage slices from the citrate fed or phosphorus injected rachitic rats was demonstrable even though there was no histological evidence of cartilage matrix calcification at the time of sacrifice.* On the basis of these observations it was suggested that, once the process of calcification in the cartilage matrix had commenced, crystals continue to be deposited despite concentrations of calcium and phosphorus in the environment too low to initiate the process. The present experiments were designed to

The original work described in this paper was carried out under a contract between the United States Atomic Energy Commission and The Johns Hopkins University.

[1] Post-Doctoral Fellow, National Institutes of Health.

[2] Research Assistant, sponsored by the United States Atomic Energy Commission.

extend these observations on the "calcifiability" of rachitic cartilage matrix following the induction of initial calcification by *in vitro* and *in vivo* techniques.

METHODS

Weanling, male, Wistar rats[3] were fed a high calcium, low phosphorus rachitogenic diet (Rachitogenic Diet #2, U.S.P. XII)[4] for 30 to 35 days. They were then sacrificed by a blow on the head, sections of the proximal tibiae were taken and immediately placed in the various inorganic solutions for incubation[5], and one cartilage slice from each rat served as a control. The details of this method of inducing calcification *in vitro* have been published previously (7) (14). Slices were first placed in the solution of high calcium concentration known to induce extensive calcification of the cartilage matrix after 24 hours incubation, but were incubated in this solution for periods varying from only ½ to 8 hours. The slices were then transferred into solutions with lower calcium concentrations for incubation during the remainder of the 24-hour period. To serve as additional controls, other slices were placed either in the solution of high or low calcium concentration for the entire 24-hour period. After incubation the slices were fixed in 95 per cent and absolute alcohol and stained with 1 per cent $AgNO_3$ for examination under a low power microscope. The amount of calcification of the cartilage matrix was graded 0 to 4+, according to a scheme presented by Marks, Hiatt and Shorr (15). It should be mentioned that, although this scheme is simple and permits good correlation among different observers, it does not represent equal increments of calcification in that 1+ indicates a thin, discontinuous line of calcification, 2+ a thin, continuous line, 3+ a broad zone of calcification across the width of the epiphysis, and 4+ indicates calcification throughout the cartilage matrix.

In the second portion of this experiment it was desired to obtain rachitic cartilage slices in which a certain amount of healing (1+ to 2+ calcification) had been induced *in vivo*. McCollum *et al.* (16) demonstrated that starvation of rachitic rats for three to five days resulted in extensive calcification of the cartilage matrix. A rise in serum phosphorus accompanies starvation and the healing effect of starvation has been believed due to this rise in serum phosphorus (17). As the amount of calcification of cartilage matrix increases with the duration of starvation, variation in the starvation period affords a means whereby controlled degrees of healing may be induced. One group of rachitic rats subjected to starvation for the 12 hours preceding sacrifice showed no evidence of calcification in the cartilage matrix, but other groups subjected to starvation for the 24 hours preceding sacrifice in-

[3] Obtained from Research Supply Co., Philadelphia, Pennsylvania.

[4] Obtained from Nutritional Biochemical Corporation, Cleveland, Ohio.

[5] The solution used for incubation was essentially that suggested by Robison (2), the final composition of which in mM. per liter is: Na 130, K 5.6, Mg 1.0, Cl 108.0, HCO_3 26.0, SO_4 1.0. To this basic salt mixture a neutral solution of Na_2HPO_4 and NaH_2PO_4 was added in the amounts required to attain an inorganic phosphorus concentration of 5.0 mg. per 100 ml. *in each incubating medium*. After this phosphorus addition the solutions were equilibrated with 5% CO_2–95% O_2 before the addition of calcium; the amount of calcium added (as calcium chloride) was varied so as to achieve concentrations of 3.0 to 8.0 mg. per 100 ml., as indicated in Tables I and II. The pH of the final solution was checked with a Beckman pH meter prior to and after incubation, the pH range observed in these experiments being 7.33 to 7.48. A minimum of 10 ml. of incubating solution was provided for each cartilage section. Incubation was carried out in *stoppered* flasks at 37.5°C., and, by use of a rocking arm, gentle agitation of the solutions was maintained throughout the period of incubation.

variably showed a 1+ to 2+ calcification of the cartilage matrix. Thus a 24-hour period of starvation was selected as the method of inducing slight healing *in vivo*. Weanling, male rats were placed on the low phosphorus rachitogenic diet for 21 to 28 days and then starved for the 24 hours (water ad libitum) prior to sacrifice. One tibial slice from each rat served as a control. The remaining slices from the proximal tibiae were incubated for 24 hours in solutions with various calcium concentrations. As additional controls, tibial slices from non-starved rachitic rats were handled in the same manner. After 24 hours incubation, the slices were fixed in 95 per cent and absolute alcohol, stained with 1 per cent $AgNO_3$ and examined under a low power microscope.

RESULTS

Part I. Table I demonstrates that when fully rachitic cartilage slices are incubated for four hours in the standard medium (containing 5.0 mg. inorganic phosphorus per 100 ml.) with a calcium concentration of 8.0 mg. per 100 ml., there occurs a 0 to 2+ calcification in the cartilage matrix.[6] When cartilage slices were incubated for 24 hours in the medium with a calcium concentration of only 4.5 mg. per 100 ml., the degree of calcification varied from 1+ to 2+. When slices were incubated for four hours at a calcium concentration of 8.0 mg. per 100 ml. and then transferred into the solution with a calcium concentration of 4.5 mg. per 100 ml. for 20 hours, the resulting calcification was invariably 3+, this degree of calcification being greater than that occurring either after four hours in the solution with a high calcium concentration or after 24 hours in the solution of low calcium concentration. Incubation of rachitic cartilage slices for 24 hours in the solution with a calcium concentration of 8.0 mg. per 100 ml. resulted in 4+ calcification of the cartilage matrix. Although the slices incubated first in the solution of high and then low calcium concentration did not show this maximum amount of calcification, the extent of calcification was appreciably greater than when the slices were incubated in the solution of low calcium concentration alone.

Additional slices (not shown in Table I) were incubated for periods of ½ to 4 hours in the solution with a calcium concentration of 8.0 mg. per 100 ml. and then incubated for the remainder of the 24 hours in solutions with calcium concentrations of only 3.0 or 3.5 mg. per 100 ml. These slices showed no increase in calcification over that which had been induced by the preliminary incubation in the solution of high calcium concentration.

In the lower portion of Table I are the observations on slices from a single rat which happened to show at the time of sacrifice slight evidence of healing *in vivo*. Slices from this rat were handled in the same manner as the others of Table I, except that the solution used in an attempt to show intensification of calcification contained only 3.5 mg. calcium per 100 ml. as compared to 4.5 mg. per 100 ml. used with the preceding slices. As reported previously (6) (7), if

[6] Although not recorded in Table I, it was found that 6 or 8 hours incubation at this calcium concentration resulted in 3+ calcification, and incubation for ½ to 2 hours resulted in 0 to 1+ calcification of the cartilage matrix.

the inorganic phosphorus concentration is maintained at 5.0 mg. per 100 ml. (as it was throughout this experiment), significant calcification *in vitro* of fully rachitic slices does not occur at such low calcium concentrations (3.5 mg. per 100 ml.). With this series of slices, where slight healing had already begun *in vivo*, extensive calcification (3+) did occur at this low calcium concentration, and preliminary incubation in the solution containing 8.0 mg. calcium

TABLE I

*Degree of Calcification in Rachitic Cartilage Slices Before and After Incubation in Inorganic Solutions Containing High and Low Calcium Concentrations**

Calcium concentration (mg. per 100 ml.) of incubating solution	Period of incubation (hr.)	Degree of calcification	Intensification of calcification
Non-incubated slices (controls)	0	0	
	0	0	
	0	0	
	0	0	
Ca = 8.0 mg.	4	1+	
	4	1+	
	4	2+	
Ca = 4.5 mg.	24	1+	
	24	2+	
	24	2+	
Ca = 8.0 mg. for 4 hr. and 4.5 mg. for 20 hr.	24	3+	Yes
	24	3+	Yes
	24	3+	Yes
	24	3+	Yes
	24	3+	Yes
	24	3+	Yes
	24	3+	Yes
Ca = 8.0 mg.	24	4+	
	24	4+	
	24	4+	
Non-incubated slice (control)	0	1+†	
Ca = 8.0 mg.	4	2+†	
Ca = 3.5 mg.	24	3+†	
Ca = 8.0 mg. for 4 hr. and 3.5 mg. for 20 hr.	24	3+†	No
	24	3+†	No
	24	3+†	No
Ca = 8.0 mg.	24	4+†	

* Inorganic phosphorus concentration 5.0 mg. per 100 ml. in each incubating medium.
† Slices from a single rat showing slight healing (1+ calcification) at time of sacrifice.

TABLE II

*Degree of Calcification in Rachitic Cartilage Slices Before and After 24 Hours Incubation in Inorganic Solutions Containing Different Calcium Concentrations**

	Non-incubated Control Slices	Incubated Slices				
		Ca-8.0 mg.%	Ca-6.0 mg.%	Ca-5.0 mg.%	Ca-4 mg.%	Ca-3.0 mg.%
Each rat starved for 24 hr. prior to sacrifice	2+	4+	3+	3+	3+	2+
	2+	4+	3+	3+	3+	2+
	1+	4+	3+	3+	3+	2+
	1+	4+	3+	3+	3+	2+
	1+	4+	3+	3+	3+	2+
	1+	4+	3+	3+	3+	2+
	2+	4+	4+	3+	3+	3+
	2+	4+	4+	3+	slice torn	3+
Control rats not starved prior to sacrifice	0	4+	3+	2+	1+	0
	0	4+	3+	0	0	0
	0	4+	3+	0	0	0
	0	4+	3+	0	0	0

* Inorganic phosphorus concentration 5.0 mg. per 100 ml. in each incubating medium.

per 100 ml. did not appear to increase this degree of calcification. This observation prompted the second portion of the experiments here reported.

Part II. In Table II are recorded the results obtained with rachitic cartilage slices showing slight healing (1+ to 2+ calcification) purposely induced *in vivo* by starvation of the rachitic rats for the 24 hours preceding sacrifice. When such slices were incubated in solutions with calcium concentrations varying from 8.0 to 3.0 mg. per 100 ml., additional calcification of the cartilage matrix occurred in the solutions with calcium concentrations of 4.0 and 5.0 mg. per 100 ml. as well as in those with higher concentrations. When cartilage slices from non-starved, control rats showing no healing *in vivo* were incubated in solutions with these calcium concentrations, minimal (0 to 2+) calcification occurred *in vitro* at calcium concentrations of 4.0 or 5.0 mg. per 100 ml., although extensive (3+ to 4+) calcification did occur in the solutions with higher calcium concentrations. Thus these observations indicate that, when calcification of cartilage matrix has been initiated by healing *in vivo*, additional calcification will occur on incubation in solutions having lower calcium concentrations than are ordinarily required to produce that degree of calcification.

DISCUSSION

These experiments indicate that once calcification of the rachitic rat cartilage has been initiated (whether by *in vivo* or *in vitro* techniques), calcification will proceed *in vitro* at lower calcium and phosphorus concentrations than are required to initiate the process or to produce the degree of calcification

finally attained. This continuation of the calcification process occurred only within a limited range of calcium concentrations. Whereas incubation of fully rachitic slices at a calcium concentration of 8.0 mg. per 100 ml. for four hours followed by 20 hours incubation at a calcium concentration of 4.5 mg. per 100 ml. did result in increased calcification, no increase in calcification occurred if the calcium concentration of the latter solution was reduced to 3.0 or 3.5 mg. per 100 ml. This same limitation occurred with the slices showing calcification which had been initiated *in vivo*, in that additional calcification occurred on 24 hours incubation at a calcium concentration of 4.0 mg. per 100 ml., but not at 3.0 mg. per 100 ml. We wish to make no inferences from the particular calcium concentrations used as to a possible solubility product for bone mineral, for no such solubility product has ever been demonstrated (18).

Possible explanations for the observations of these experiments are the physico-chemical reactions occurring between crystals and the ions of a surrounding solution. Greenwald (19) has emphasized that it is the extent of surface of the solid phase that is important in determining the equilibrium finally attained between the ions of the solid phase and the ions in simple solution. Studying the equilibrium between a precipitate of calcium and phosphorus and these ions in solution, he showed that with increasing size (and surface area) of the solid phase, there was an increasing amount of calcium and phosphorus ions remaining in solution at equilibrium. These observations on the reactions between crystals and ions in solution have been extended and amplified by Hodge (20). To apply them to the present experiments would suggest that, with the increase in calcification of the cartilage matrix, there is a decrease in crystalline surface area that is exposed to the ions of the incubating solution. This suggestion is in keeping with the conclusion of Neuman (21), who in discussing bone crystals has stated that ". . . the crystals are in such intimate contact (perhaps by actual bond formation) with the organic phase that much of the crystalline surface is unexposed . . ." Furthermore, Kuyper (22), while investigating the solubility product of calcium and phosphorus as it might apply to bone crystals, made some observations which seem applicable to the present experiments. Using inorganic solutions comparable to serum ultrafiltrate except for somewhat higher calcium and phosphorus concentrations, he showed that once a precipitate (solid phase) formed, there was continued increment to the solid phase until equilibrium was reached between the solution and the solid phase despite the lowering of the calcium and phosphorus concentrations in the surrounding solution to values *below that initially required for precipitation*. The observations of the present experiments on this one type of biological calcification suggest that physico-chemical reactions similar to those reported by Kuyper occur during calcification of rachitic cartilage matrix.

The possible pertinence of these observations to the healing process of child-

hood and adult (osteomalacia) rickets in man should be considered. Despite the probable varying composition of bone and cartilage apatites (21) and the purely hypothetical nature of a solubility product for (Ca^{++}) and ($PO_4^{=}$) in biological fluids (20) (21), nevertheless the postulate introduced by Howland and Kramer (23) has held good through the years with remarkable consistency: namely that, when the "product" of serum Ca x P is less than 30, histologically evident rickets of both cartilage and bone will be found, and that when the "product" is above 40, either no rickets or healing rickets will be found. The required "product" will, of course, vary at different ages, being greater in infants and decreasing toward adult life (24).[7] Exceptions to this rule occur, so far as is known, only in renal insufficiency, where it is believed some unknown constituent of the serum interferes with the mechanism of calcification (7), and in certain instances of rapid growth of new bone such as about healing fracture sites.[8]

When healing of rickets occurs *in vivo*, both cartilage and osteoid calcify. Yet calcification *in vitro* of rachitic osteoid has not been reported either in experimentally produced rickets or in that occurring naturally. Partly for this reason, it was recently suggested (28) that bone matrix, in contradistinction to cartilage matrix, may be enclosed in a membrane across which a chemical gradient exists; this membrane being composed of osteoblasts and their extensions and under control, therefore, of enzymes of the osteoblasts.

In human rickets administration of vitamin D in adequate amounts usually is followed by an increase of the phosphorus or calcium (or both) in the serum and healing ensues. Also healing of rickets has been observed to proceed normally during administration of citric acid and citrate mixtures even while serum calcium and phosphorus were depressed below the initial concentrations (11). Yendt *et al.* (14) observed after citrate administration to rachitic rats (rickets induced by low phosphorus diet) that there was an early rise in serum phosphorus. The observations here presented suggest that the initial phosphorus rise observed by Yendt may have been sufficient to initiate the process of calcification, which then might proceed even though the serum phosphorus had soon receded to its previously low concentration.

One also meets, rarely, instances of rickets or osteomalacia which are seem-

[7] It is the extracellular concentrations of calcium and phosphorus that are of importance in determining the presence or absence of rickets. As the calcium and phosphorus concentrations of serum ultrafiltrates provide a measure of extracellular calcium and phosphorus, the required serum "product" will, therefore, vary with alterations of serum proteins (25) and of other substances which chelate calcium or phosphorus (14), thus affecting the availability of these ions for calcification.

[8] More than normal amounts of osteoid are sometimes seen in severe thyrotoxicosis (26), about local skeletal lesions of myeloma, leukemia and metastatic carcinoma, and occurs in animals receiving strontium, excess vitamin D, etc. (27).

ingly due to phosphate wastage by the kidney with resultant low serum phosphorus (29) (30). If the experimental observations reported in this paper are valid for the human with rickets resistant to ordinary therapy, one might approach this difficult therapeutic problem by measures which would repeatedly raise the serum phosphorus for brief periods of time. Intravenous infusion of phosphate or heavy dietary loading with phosphates for short periods might accomplish a benefit to the skeleton far beyond the short duration of the effects evident by alterations of the serum.

SUMMARY

Using standard techniques for the study of calcification *in vitro* of rachitic rat cartilage, the experiments reported here are believed to demonstrate that, once deposition of mineral apatite has been initiated, further deposition will occur even in solutions containing lower concentrations of calcium and phosphorus than are necessary for initiation of the process. This was demonstrated by: (1) initiation of calcification *in vitro* (using for brief periods of time concentrations of calcium and phosphorus known to produce calcification and following this by incubation in solutions of lower calcium concentrations); and (2) after inducing slight calcification *in vivo*, finding that calcification would continue *in vitro* in solutions with concentrations of calcium and phosphorus which would not have produced calcification in fully rachitic cartilage.

Possible application of these observations to the healing process in human rickets is discussed.

REFERENCES

1. Robison, R.: XXXIII. The possible significance of hexosephosphoric esters in ossification. Biochem. J., 1923, **17**: 286.
2. Robison, R. and Rosenheim, A. H.: XCV. Calcification of hypertrophic cartilage in vitro. Biochem. J., 1934, **28**: 684.
3. Gutman, A. B. and Yu, T. F.: Further studies on the relation between glycogenolysis and calcification in cartilage. *Metabolic Interrelations*. Tr. First Conf., sponsored by The Josiah Macy, Jr. Foundation, New York, N. Y., 1949, p. 11.
4. Goldenberg, H. and Sobel, A. E.: Calcification. V. Influence of fluoride and cyanide ions in the presence and absence of magnesium. Proc. Soc. Exper. Biol. & Med., 1951, **78**: 719.
5. Hiatt, H. H., Marks, P. A. and Shorr, E.: Effects of inhibitors on calcium deposition in cartilage in vitro. J. Biol. Chem., 1953, **204**: 187.
6. Sobel, A. E., Goldenberg, H. and Schmerzler, E.: Calcification. XI. Studies of the incorporation of citrate in calcification in vitro. J. Dent. Research, 1954, **33**: 497.
7. Yendt, E. R., Connor, T. B. and Howard, J. E.: In vitro calcification of rachitic rat cartilage in normal and pathological human sera with some observations on the pathogenesis of renal rickets. Bull. Johns Hopkins Hosp., 1955, **96**: 1.
8. Shohl, A. T. and Butler, A. M.: Citrates in the treatment of infantile rickets. New England J. Med., 1939, **220**: 515.
9. Rominger, E.: Ueber versuche zur rachitisheilung und verbrütung ohne von licht anwendung oder D-vitamin. Arch. Kinderh., 1944, **131**: 53.

10. ELIOT, M. M. AND PARK, E. A.: *Brenneman's Practice of Pediatrics*, W. F. Prior Co., Inc., Hagerstown, Md., 1948, Vol. I, chapter 36.
11. HARRISON, H. E. AND HARRISON, H. C.: Further studies of the effects of citrate feeding on the calcium, phosphorus and citrate metabolism of rachitic infants. J. Pediat., 1952, **41:** 756.
12. HAMILTON, B. AND DEWAR, M.: Effect of citrate and tartrate on experimental rickets. Am. J. Dis. Child., 1937, **54:** 548.
13. SHOHL, A. T.: The effect of acid-base content of the diet upon the production and cure of rickets with special reference to citrates. J. Nutrition, 1937, **14:** 69.
14. YENDT, E. R. AND HOWARD, J. E.: Studies on the mode of action of citrate therapy in rickets. Bull. Johns Hopkins Hosp., 1955, **96:** 101.
15. MARKS, P. A., HIATT, H. H. AND SHORR, E.: Factors influencing deposition of calcium and strontium in cartilage in vitro. J. Biol. Chem., 1953, **204:** 175.
16. MCCOLLUM, E. V., SIMMONDS, N., SHIPLEY, P. G. AND PARK, E. A.: Studies on experimental rickets. XV. The effect of starvation on the healing of rickets. Bull. Johns Hopkins Hosp., 1922, **33:** 31.
17. PARK, E. A.: The influence of severe illness on rickets. Arch. Dis. Childhood, 1954, **29:** 369.
18. HODGE, H. C.: Some observations on the dynamics of calcification. *Metabolic Interrelations*. Tr. Second Conf., sponsored by The Josiah Macy, Jr. Foundation, New York, N. Y., 1950, p. 73.
19. GREENWALD, I.: The solubility of calcium phosphate. I. The effect of pH and of amount of solid phase. J. Biol. Chem., 1942, **143:** 703.
20. HODGE, H. C.: Some considerations of the solubility of calcium phosphate. *Metabolic Interrelations*. Tr. Third Conf., sponsored by The Josiah Macy, Jr. Foundation, New York, N. Y., 1951, p. 190.
21. NEUMAN, W. F. AND NEUMAN, M. W.: The nature of the mineral phase of bone. Chem. Rev., 1953, **53:** 1.
22. KUYPER, A. C.: The chemistry of bone formation. II. Some factors which affect the solubility of calcium phosphate in blood serum. J. Biol. Chem., 1945, **159:** 417.
23. HOWLAND, J. AMD KRAMER, B.: A study of the calcium and inorganic phosphorus of the serum in relation to rickets and tetany. Monatschr. Kinderh., 1923, **25:** 279.
24. HOWARD, J. E.: Discussion, *Metabolic Interrelations*, Tr. Fourth Conf. sponsored by The Josiah Macy, Jr. Foundation, New York, N. Y., 1952, p. 149.
25. HOPKINS, T. R., CONNOR, T. B. AND HOWARD, J. E.: Ultrafiltration studies on calcium and phosphorus in pathological human serum. Bull. Johns Hopkins Hosp., 1953, **93:** 249.
26. FOLLIS, R. H.: Skeletal changes associated with hyperthyroidism. Bull. Johns Hopkins Hosp., 1953, **92:** 405.
27. FOLLIS, R. H.: Diseases, particularly of bone, associated with derangements of calcium and phosphorus metabolism. *Metabolic Interrelations*, Tr. Fifth Conf., sponsored by The Josiah Macy, Jr. Foundation, New York, N. Y., 1953, p. 196.
28. HOWARD, J. E.: Present knowledge of parathyroid function, with especial emphasis upon its limitations. Tr. Conf. on *Bone Structure and Metabolism*, sponsored by The Ciba Foundation, London, England, 1955, in press.
29. ALBRIGHT, F., BUTLER, A. M. AND BLOOMBERG, E.: Rickets resistant to vitamin D therapy. Am. J. Dis. Child., 1937, **54:** 529.
30. PARK, E. A.: The therapy of rickets. J.A.M.A., 1940, **115:** 370.

TUBERCLE BACILLUS BACTERIAL ENDOCARDITIS DEVELOPED UPON A SURGICAL MITRAL "SLING"

R. PATTERSON RUSSELL,[1] MORGAN BERTHRONG AND AUTREY R. CROKE

The Departments of Pathology and Internal Medicine, The Glockner-Penrose Hospital, Colorado Springs, Colorado

Received for publication June 13, 1956

Tubercle bacillus bacterial endocarditis, in the broadest sense, indicates infection of the endocardium or valves of the heart by tubercle bacilli whatever their source. During massive hematogenous spread of tubercle bacilli, tiny tubercles identical to those formed in other organs may develop in the endocardium of the cardiac chambers or, more rarely, on the cardiac valves. Many years ago, this was said to occur as often as in nine of every ten cases of acute miliary tuberculosis (1), but it has not occurred or at least has not been recognized that often in more recent years. A second variety of tuberculous infection of the endocardium is the result of direct invasion from lesions in the mediastinum, pericardium or myocardium. These are usually caseous masses which project into a chamber or even into a valve of the heart. Such lesions may occasionally result in hematogenous spread of the bacilli or, rarely, in malfunction of the valve. Neither the endocardial tubercles of miliary tuberculosis nor the extension of tuberculous masses from without are analogous to the usual case of either acute or subacute bacterial endocarditis caused by other organisms. It seems wise to consider such cases, as Baker suggested (2), "endocardial tuberculosis", since the term "tuberculous endocarditis" implies a more specific entity.

Only a very rare case has been described in which tubercle bacilli multiplied upon or caused vegetations of either diseased or normal cardiac valves or on the endocardium, thus to be thought similar to endocarditis produced by other bacteria. Yet, if one considers how common hematogenous dissemination of tubercle bacilli in patients with either normal or abnormal valves must be, the rarity of true tubercle bacillus bacterial endocarditis is peculiar.

Baker in an excellent review of the literature prior to 1935 (2), could accept only four cases, and three of these were poor examples. His criteria included: the presence of tubercle bacilli and absence of other organisms; lesions not simply part of hematogenous tuberculosis nor of an extension of tuberculosis. In 1936 Davie (3) described two patients with mitral valvular vegetations similar to other bacterial vegetations, but in which only acid-fast bacilli were found. These vegetations occurred on normal valves but Davie postulated that non-bacterial fibrin deposits may have antedated and predisposed to the tuber-

[1] Now intern, Department of Medicine, Johns Hopkins University and Hospital.

cle bacillus infection. Mark (4) added an acceptable case in 1938, the infection occurring on the pulmonary valve of a child. He stated that it was the only case which had been seen in nearly 16,000 autopsies at the Johns Hopkins Hospital. In this instance, also, the bacilli had infected a previously normal valve. Gilmore in 1940 (5) reported a tuberculous endocarditis of the pulmonary valve, and in 1950 Baron and Ritter (6) described the tubercle bacillus infection of a large, thrombotic mass in the left auricular appendage of a patient with heart failure. This patient had tubercle bacilli infected infarcts of the brain and spleen. All of these patients described with true tubercle bacillus bacterial endocarditis have had miliary tuberculosis as a consequence of the disease.

It is in view of the extraordinary rarity of authentic tubercle bacillus bacterial endocarditis that we wish to present the following case, one all the more unusual in that the infection occurred on an abnormal valve.

CASE REPORT

The patient was a 31-year-old white woman who was admitted for the last time to the Glockner-Penrose Hospital on June 25, 1954, for fever, weakness and weight loss.

Family history: Her mother and father were both living and well. One cousin had died of a congenital heart disease at the age of 16 years.

Past history: She had had the usual childhood diseases. From 1934 to 1936 she had been confined to bed with "St. Vitus dance", followed by joint and cardiac manifestations.

Present illness: She had been known to have an enlarged heart and cardiac murmurs for years, but was able to pursue normal activities until 1949, when cardiac failure first appeared. This and immediate subsequent attacks thought to be entirely the consequence of mitral regurgitation were controlled with digitalis, diuretics and salt restriction. In 1951, auricular fibrillation was noted and shortly thereafter a cerebral embolus resulted in a partial left hemiparesis. In February, 1952, largely at the patient's insistence that "something be done", she had a transventricular pericardial tamponage performed by Dr. Charles Bailey of Philadelphia. A tube of pericardium was inserted across the ventricular side of the mitral valve orifice through small incisions in the anterior and posterior left ventricular wall. At operation, it was thought that this "sling" reduced the regurgitation by 85 per cent.

Following the operation very little objective improvement in her cardiac status occurred and she was repeatedly admitted to the hospital for congestive failure, auricular fibrillation, and episodes of pulmonary embolization. In July of 1953 she was given radioactive iodine to induce hypothyroidism. In August of 1953 congestive failure was again present and petechiae of the skin were noted. No fever or leukocytosis was present, but three blood cultures revealed a hemolytic Staphylococcus aureus. Three and six-tenth million units of penicillin a day were given for six weeks and blood cultures were consistently negative thereafter. She returned in November of 1953 with a second outbreak of petechiae associated with weakness and anorexia. In spite of a normal white blood cell count and the absence of fever, hemolytic Staphylococcus aureus was again cultured from the blood. She was apparently cured by six million units of penicillin per day for six weeks.

Two months later, in February, 1954, she was re-admitted for treatment of congestive heart failure but showed no evidence of bacterial endocarditis. She had an attack of urinary tract infection in May of 1954, controlled with Gantrisin.

Physical examination: Temperature, 104°F.; pulse, 88; respiration, 22; blood pressure,

110/60. She was a slender, pale asthenic white woman obviously desperately ill. The skin was free of petechiae. No splinter hemorrhages were noted beneath the nails. The fundi were clear. No distention of neck veins was present. The lungs were clear to percussion and auscultation. The heart was enormously enlarged with a booming P_2 and a grade IV apical systolic murmur. A faint diastolic murmur was audible. These were the same murmurs heard on recent previous admissions. The liver was felt two finger breadths below the costal margin. The spleen was enlarged to percussion, but was not felt. There was no peripheral edema.

Laboratory: The hemoglobin was 11 gm. The white blood cell count was 8,150 with 64 segs., 2 stabs., 31 lymphs., and 3 basos. The urine had a specific gravity of 1.008 and was negative for albumen and sugar. The sediment was loaded with white cells. Four blood cultures were negative. The sputum contained normal flora by routine cultures. Agglutinations for Brucella, Paratyphoid, and Proteus O, H, and OX were all negative. Total blood protein, albumen-globulin ratio, and cephalin flocculation tests were normal. The electrocardiogram showed auricular fibrillation, digitalis effect and probable ischemic myocardial changes.

X-rays: The heart was huge, with a large left atrium displacing the left main bronchus and esophagus. The lung fields showed numerous small nodules, 1 to 3 mm. in diameter, most numerous in the perihilar zones, but extending to both apices and bases. It was thought that most of the nodularities had developed within six weeks. Hemosiderosis, congestive failure, or miliary tuberculosis were suggested diagnoses.

Course: The fever fluctuated in a typical septic fashion between 101° and 104°. The spleen became palpable. No petechiae were ever seen. The white blood cell count rose to 18,000 and showed a shift to the left. She was given intravenous penicillin, oral streptomycin, and a blood transfusion but died four days after admission.

Autopsy Number 54A-64: The autopsy was performed eighteen hours after death on a body previously embalmed by arterial injection. The pertinent findings follow.

External appearance: The body was that of a well developed, well nourished, white female, measuring 69 inches in length and weighing approximately 120 pounds. No jaundice or edema was present. There was a well-healed scar on the chest following the fifth left interspace and extending from beneath the left breast to the back measuring 25 cm. in length. The legs measured:

Right—7¼–9¾–11½–13¼–14½ inches in circumference;

Left—7¼–9¾–11½–13½–14¾ inches in circumference.

Abdominal cavity: There was approximately 300 cc. of straw-colored fluid in the abdominal cavity. The peritoneal surfaces were glistening and free from fibrous adhesions. The abdominal organs were normal in color and location.

Thoracic cavity and mediastinum: There were numerous fibrous tags binding the pleurae to the walls of the pleural cavities. The pleura of the left lung was firmly adherent to the lateral chest wall. The pericardium was also tightly adherent to the left chest wall and to the epicardium, obliterating the pericardial cavity. The thymus was atrophic.

Heart: The heart weighed between 950 and 1050 gm., but the pericardium and a small portion of the diaphragm were included in this weight. The adhe-

sions were so firm to the left chest wall that sharp dissection had left some myocardium attached to the ribs. The heart showed hypertrophy and dilatation of all chambers with extreme dilatation of the left atrium and ventricle. The foramen ovale was sealed closed as was the ductus arteriosus. The tricuspid valve leaflets were thickened and rolled along the free margins. The chordae tendineae were shortened and thickened. The pulmonary valve was dilated. The mitral valve leaflets were thickened and rolled along the margins and the entire posterior leaflet was scarred and contracted. The chordae tendineae, especially of the latter leaflet, were shortened and thickened. Extending across the upper portion of the left ventricular chamber and in part fused with the under surfaces of the mitral valve leaflets there was a cord-like hyaline mass, 8 cm. in length and 2 cm. in diameter. The posterior insertion of this cord was near the posterior junction of the mitral valve leaflets. The cord could be traced as a much thinner strand of dense, fibrous tissue which extended through the myocardium and was imbedded in the posterior left ventricular epicardium for a distance of 2 cm. Anteriorly, the cord was inserted into the left ventricular myocardium at the anterior junction of the mitral valve leaflets again forming a dense, fibrous band 0.75 cm. in diameter, which passed through the myocardium and then fanned out in the dense pericardial scars. The total length of the cord from anterior to posterior epicardial insertions was 16 cm. The "sling" virtually filled the mitral valve orifice (Fig. 1). It was very rigid and surely caused mitral stenosis since a finger could be inserted with difficulty past the "sling" through the valve ring from above. The rigid fixation of the "sling" probably also prevented significant regurgitation. The surface of this "sling" was smooth, save for several firm, fibrous, pedunculated vegetations, varying in size from 0.2 to 0.5 cm., which were attached to the inferior surfaces of the intraventricular portion of the "sling" (Figs. 2 & 3). One polypoid, friable, smooth vegetation was hanging down from its attachment deep in a crypt between the sling and the posterior papillary muscle (Figs. 2 & 3). This measured 1.5 by 0.5 cm. The aortic valves were thickened, rigid, and partially calcified. There did not appear to be any insufficiency of the aortic valve. The valve rings measured: tricuspid, 13 cm.; pulmonary, 8.5 cm.; mitral, 10 cm.; aortic, 6.5 cm. The right ventricular myocardium measured 6 mm. in thickness and showed very prominent trabeculae carneae. The left ventricular myocardium measured 15 mm. in thickness. The left ventricle was extremely dilated. There was no evidence of myocardial infarction. The coronary ostia were patent though somewhat small. The coronary arteries were patent, with an occasional small, arteriosclerotic plaque. The epicardial insertion of the pericardial "sling" did not damage large coronary artery branches.

Lungs: The pleurae were rough and adherent to the walls of the pleural cavities in many places. The pleurae within interlobar fissures were fused with fibrous tissue. The lungs were large and were firm in consistency. On section the

parenchyma of the lungs was studded with small, grey nodules, measuring from 1 to 2 mm. in diameter. An encapsulated, grey-yellow nodule, measuring 2 by 2 by 3 cm., with a mottled, pink, soft center, was found at the lower border of the right upper lobe several centimeters from the pleural surface. The pulmonary veins appeared dilated near their entrance into the left atrium. The pulmonary artery revealed slight arteriosclerotic plaque formation.

Liver: The liver weighed 1400 gm. The capsule was smooth and glistening. On section, yellowish streaks were present throughout the liver parenchyma. The normal lobular architecture was disturbed by small nodules 2 to 3 mm. in diameter, with dark brown central areas surrounded by the yellowish streaks. The portal vein was patent and contained no thrombi.

Spleen: The spleen weighed 530 gm. It was firm in consistency and on section the pulp was dark red. The malpighian bodies and trabeculae were distinct. The splenic vein was patent and contained no thrombi.

Adrenals: The right adrenal weighed 8 gm., and the left weighed 9.5 gm. On section the adrenals contained small foci of lipoid depletion interspersed among areas of yellow cortical substance. No other abnormalities were present.

Kidneys: The right and left kidneys each weighed 150 gm. The capsules stripped with ease, revealing a "flea bitten" surface. The cortices and pyramids showed no abnormalities. The calices, pelves and ureters were normal. A small probe freely entered the bladder from each ureter.

Other viscera, including brain: No gross abnormalities were seen.

Microscopic examination: Sections of the left ventricle disclosed old and recent vegetations arising from the undersurface of the "sling", as well as developing from the crypts lying between the "sling" and the papillary muscles. Histological study of the old vegetations showed hyaline scarring, while sections of the large friable vegetation revealed acellular fibrin which was partially hyaline and partially necrotic, with fibrous organization and granulation tissue only at its base (Fig. 4). Kinyoun acid-fast stains of this vegetation showed enormous numbers of acid-fast bacilli lying singly and in colonies, often forming distinct cords, throughout all areas of the vegetation (Figs. 5 & 6). No tubercles or giant cells were present. Indeed, where bacilli were most numerous no inflammation of any kind was seen in the amorphous material (Fig. 5). Bacterial stains revealed these organisms to be both Gram negative and Gram positive curved, beaded bacilli, but did not show any other bacteria. Stains of the older vegetations did not show organisms. Between the infected vegetation and the papillary muscle there was a subacute inflammation of the opposing endothelial surfaces, but there were no tubercles or giant cells. Elastic tissue stains of this area showed an intact elastic membrane between the vegetation and the myocardium. Tiny tubercles, typical of miliary tuberculosis, were seen in the epicardium and endocardium in other sections of the left ventricle. The

tricuspid valve showed slight scarring. No Aschoff bodies were seen in the myocardium or endocardium of many sections of all chambers of the heart.

Numerous sections of the lung revealed small, uniformly distributed tubercles, composed of epithelioid cells and Langhans giant cells, with caseous centers (Fig. 8). The Kinyoun acid-fast stains revealed occasional acid-fast bacilli in these tubercles. The encapsulated caseous mass several centimeters in diameter was entirely typical of older primary tuberculosis (Fig. 7). The fibrous capsule of this focus was thin, and in several areas only thin layers of connective tissue separated the caseous center from lumina of adjacent veins. The Kinyoun acid-fast stain revealed acid-fast bacilli in the center of the tubercle. Sections of the liver revealed scars which appeared to arise in the central areas, radiating into portal regions. There were also numerous, small, epithelioid cell tubercles with giant cells scattered in the liver parenchyma and portal areas. Sections of the spleen showed small, uniformly distributed miliary tubercles in which acid-fast bacilli were stained. Sections of the kidney showed several foci of caseation and Langhans giant cells. Kinyoun acid-fast stain failed to reveal acid-fast bacilli in the renal lesions. Periaortic lymph nodes showed many small miliary tubercles. Sections of the pancreas revealed chronic passive congestion. The bone marrow also contained miliary tubercles and acid-fast stains showed acid-fast bacilli. The thyroid showed scarring and atypical cellular hyperplasia.

DISCUSSION

We believe that this patient's terribly malformed mitral valve and the surgically created "sling" became repeatedly infected and cured of a common bacterial organism. Tubercle bacilli from the older pulmonary lesion then probably were implanted on a vegetation and slowly multiplied into enormous numbers. The patient's final acute and fatal episode was the result of a massive hematogenous spread, most likely from the valvular tuberculosis.

From the criteria for the diagnosis of tubercle bacillus bacterial endocarditis already listed and originally formulated by Baker, our case and several others in the literature are deficient only in respect to the tissue reaction. We suggest, however, that when tubercle bacilli reach a valve through small blood vessels, an inflammatory reaction is possible in the vascular area and the tissue changes of tuberculosis develop. The cases described by Mark (4), Gilmore (5), and Baron (6) are ones where, since the bacilli were multiplying in areas of adequate blood supply, typical tubercles formed. It would seem likely, however, that when bacilli are implanted upon an avascular valve surface or in fibrinous vegetations, whether rheumatic or bacterial in origin, the organisms may multiply in the area without inciting a typical tissue reaction. Such an absence of tissue response is indeed found in other varieties of bacterial endocarditis except at the very base of the vegetations. Especially in tuberculosis,

where the characteristic tissue reaction depends so much upon the presence of either tissue or blood mononuclear phagocytes, a deficient blood supply may limit the usual tissue response. Thus, other cases of true tubercle bacillus endocarditis have shown only very weak or actually no typical inflammatory response, as in Meek's (7) and in Davie's (3) cases and in our own.

All other aspects of this case strongly indicate it as one of the true tubercle bacillus bacterial endocarditis. The huge colonies of typically acid-fast bacilli, growing in "cord formations" deep in the acellular vegetations had probably been present for many weeks before the occurrence of miliary tuberculosis. These colonies are reminiscent of those of pneumococci in leukopenic rabbits (8) and might suggest the failure of antibodies to control multiplication of tubercle bacilli in the absence of monocytes. Excellent and controlled bacterial stains have failed to demonstrate other organisms in the vegetation.

The development of a tuberculous vegetation upon a previously damaged valve and possibly a pre-existing vegetation is unique. (Table I). Davie could only postulate that tuberculous endocarditis in his patients had developed in a pre-existing non-bacterial endocarditis for no Aschoff bodies were present in the myocardium of his cases and no healed vegetations were seen as in our patient. It may be noted in Table I that of the eight cases presented the tuberculous lesion was found on the left side of the heart in six instances.

Several other points of interest in our patient may be briefly mentioned. First, in spite of what was certainly severe mitral stenosis, typical miliary tuberculosis in the lungs developed. The supposed infrequency of this event has been repeatedly mentioned in textbooks. Second, an incredibly difficult surgical procedure probably corrected the previous insufficiency, but added a serious mitral stenosis.

TABLE I

Cardiac Lesions in Eight Authentic Cases of Tubercle Bacillus Bacterial Endocarditis

Author	Location	Previous State of Endocardium		Previous Vegetations	Tuberculous Tissue Reaction	Source of Bacilli	Terminal Miliary Tuberculosis
		Normal	Diseased				
Meek	Aortic and Mitral Valves	+	−	?	+ −	Kidney or bone	+
Meek	Mitral Valve	+	−	?	0	Peritoneum	+
Davie	Mitral Valve	+	−	0	0	Kidney or bone	+
Davie	Mitral Valve	+	−	0	0	Kidney	+
Mark	Pulmonary Valve	+	−	0	+	Disseminated Tuberculosis	+
Gilmore	Pulmonary Valve	+	−	0	+	Disseminated Tuberculosis	+
Baron	Left Auricular Appendage	?	?	?	+	?	+
Russell	Mitral Valve	−	+	+	0	Lung Encapsulated Primary Focus	+

SUMMARY

A case of tuberculous endocarditis is presented which developed upon the mitral "sling" of a surgically treated mitral insufficiency. The tubercle bacilli may have lodged upon the vegetation of a previous, cured bacterial endocarditis. Typical miliary tuberculosis developed terminally.

BIBLIOGRAPHY

1. WEIGART: Zur Lehre von der Tuberculose und von verwandten Erkrankungen. Virchows Arch. Path. Anat., 1879, **77,** 269.
2. BAKER, R. D.: Endocardial tuberculosis. Arch. Path., 1935, **19,** 611.
3. DAVIE, T. B.: Tuberculosis endocarditis. J. Path. and Bact., 1936, **43,** 313.
4. MARK, J.: Tuberculous endocarditis of the pulmonary valve. Bull. Johns Hopkins Hosp., 1938, **63,** 415.
5. GILMORE, H. R.: Tuberculosis involving the pulmonary valve. Am. J. Path., 1940, **16,** 229.
6. BARRON, E. AND RITTER, D. W.: Endocardial tuberculosis. Ann. Int. Med., 1950, **33,** 1023.
7. MEEK, W. O.: Tuberculous endocarditis. Proc. Roy. Soc. Med., 1908, **1,** 116.
8. RICH, A. R. AND MCKEE, C. M.: A study of the character and degree of protection afforded by the immune state independently of the leucocytes. Bull. Johns Hopkins Hosp., 1934, **54,** 277.

Explanation of Illustrations

Fig. 1. Dilated left atrium with filling of mitral orifice by rigid pericardial "sling".

Fig. 2. Trabeculated left ventricle with old vegetations on right and fresh vegetation on left (arrow) beneath and near the point of insertion of the "sling".

Fig. 3. The sling extending across mitral valve ring showing old fibrous vegetations (above label) and fresh polypoid vegetation on right. The latter contained the tubercle bacilli. Note the huge left atrium.

Fig. 4. Section through the base of the large friable vegetation with fibrous organization and granulation tissue (low power).

Fig. 5. Section of the same vegetation showing colonies of tubercle bacilli (acid-fast stain) (low power).

Fig. 6. High power magnification of tubercle bacilli. Note the distinct cord formation (acid-fast stain) (oil immersion).

Fig. 7. Encapsulated caseous mass in the lung parenchyma typical of an encapsulated primary tuberculosis (low power).

Fig. 8. Section of lung parenchyma with several small tubercles containing epithelioid cells, Langhans giant cells, and caseous centers (low power).

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

FIG. 5.

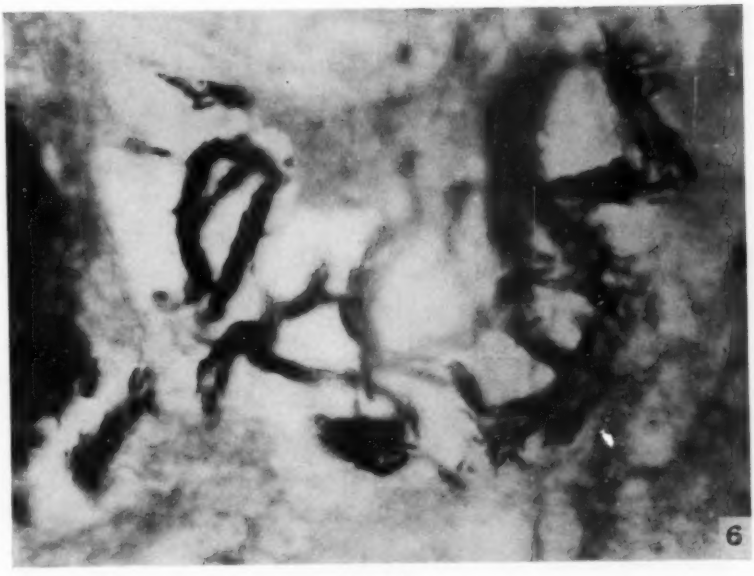


FIG. 6.

FIG. 7.

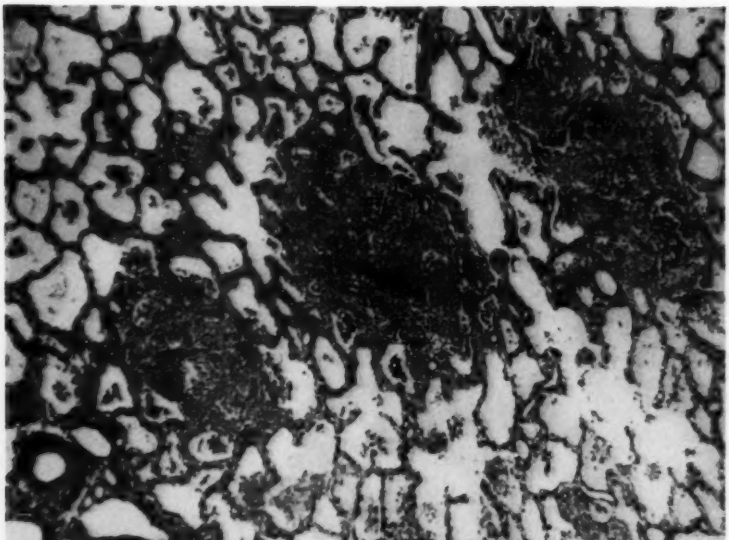
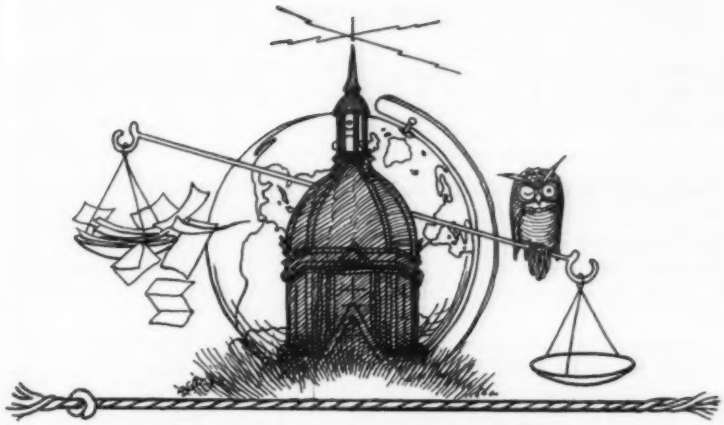


FIG. 8



BOOK REVIEWS

Diseases of the Skin, 11th edition. By RICHARD L. SUTTON, JR. 1479 pp., $29.50 The C. V Mosby Co., St. Louis, Missouri.

Dermatologists throughout the English speaking world have looked forward to each new edition of *Diseases of the Skin* since it first appeared in 1916. Over the years this magnificent text has been enlarged and improved as the result of the dedicated and increasing labors of a talented father and a talented son. The reviewer has always regarded it as the most useful book on dermatology because it is comprehensive and detailed, and because it contains the best bibliography of the standard texts. In the author's own words: "The book includes features of a dictionary and an encyclopedia. It is a guide to the maze of what may be found, if one knows where to look, in an extensive medical library. It is a systemization of what is known about its subject, written from the viewpoint of a practicing physician who is inquisitive." There are 1972 illustrations and 1479 pages of text. To avoid publishing his material in two volumes the author used 8 point type for material of secondary importance.

There are really several books within a book. The chapter on dermatoses due to fungi and the section dealing with syphilis could be separately published as very creditable books. The material concerned with the "basic subjects" of embryology, anatomy and physiology has been revised to include the numerous recent advances in these fields. The chapter on chemical agents and drugs is up to date and includes an excellent discussion of the use of steroids in dermatology. Perhaps the least distinguished chapter is the one on mechanical and physical treatment agents. One adverse criticism I would make is Sutton's insistence on the value of the actual cautery as an instrument of precision, and his casual discussion (in 3 brief paragraphs) of high frequency and fulguration currents. The discussions on clinical dermatology and pathology are masterful. Some of the author's views of therapy may be considered empirical by others but the author makes no apologies for the fact that at times he is an empiricist. His views on acne, particularly in regard to the influence of thyroid (p. 690), are well known and frequently contested, but he persists in them. As his father was before him, Richard Sutton, Jr. is a forceful, attractive, imaginative individualist and his personality is clearly exhibited throughout this fine book. Interspersed with the mass of facts which represents a summary of dermatologic dogma there will be found many interesting original comments, personal illusions and opinionated disagreements regarding some accepted practices.

The speciality of dermatology and the medical profession at large are indebted to the Suttons for their years of devotion to the task of presenting an up-to-date authorative treatise on *Diseases of the Skin.*

Maurice Sullivan

Die Chirurgie der Peripheren Nerven. By HENRY NIGST, with foreword by RUDOLF NISSEN. 196 pp., Ganzleinen DM 29.70 ($7.10). Georg Thieme Verlag, Stuttgart, Germany. Also Intercontinental Medical Book Corp., New York, N. Y.

This monograph gives an excellent up-to-date review of the problem of peripheral nerve surgery. An outstanding quality of the book is the precise and clear-cut presentation of the material.

In a general part the author summarizes the most important diagnostic signs of the various nerve lesions and briefly describes the current neurophysiological aspects. There is an excellent and most consciencious chapter regarding the operative indications, mainly based upon the experiences of the author with Professor Seddon in London. The directions for the operative technique and postoperative care are clear and understandable.

Dr. Nigst gives us a good review of the pathological conditions which can be responsible for peripheral nerve injury lesions of traumatic origin. The general surgeon, the orthopedist or neurosurgeon will profit greatly from the well-illustrated special part, in which the author analyzes the characteristics of each important nerve lesion.

There is an extensive bibliography, which includes practically all of the important contributions from the European and North American clinics.

George W. Smith

Essentials of Dermatology, 5th edition. By NORMAN TOBIAS. 651 pp., $8.00. J. B. Lippincott Co., Philadelphia, Pa.

This is one of the best of the numerous "smaller books" on dermatology. So great has been the development of dermatology in the past two decades, that small books dealing with the "bare essentials" of the subject are no longer small. In this book of 651 pages there is space only for brief summaries, which are completely and attractively presented. There is a great demand for this type of book particularly from medical students who are harassed by schedules which permit minimal exposure to an important discipline of medicine. This book will serve the student as an excellent outline, but it should be used in conjunction with more comprehensive texts. The particular feature of *Essentials of Dermatology,* which sets it apart for special tribute, is the consideration of the nursing aspects of skin diseases. At the end of each chapter the author describes the treatment technics which are entrusted to nurses in the hospital and the office. The reviewer enthusiastically recommends Tobias' book to all Schools of Nursing as an excellent standard required text.

Maurice Sullivan

Electrodiagnosis and Electromyography. Vol. 1 of Physical Medicine Library. Edited by SIDNEY LICHT. 272 pp., $10.00. Elizabeth Licht, Publisher, New Haven, Conn.

Electrodiagnosis refers to diagnostic procedures based on the introduction or removal of electric currents. Its most successful applications have been the recording of the rhythmic and easily standardized potentials continuously evolved by the heart, and the less regular potentials produced by the brain. Much more difficult has been the clinical application of information obtained by recording the potentials which arise in striated muscle during voluntary or electrically induced activity (electromyography), or by observing the response of muscle to electrical stimulation of motor nerve or muscle (muscle electrodiagnosis). Dr Licht, and the other contributors to this book, which is intended to be the first volume of a

"physical medicine library", have presented an interesting summary of the historical development of the latter procedures, the apparatus and methods employed, and their limited clinical application. Muscle electrodiagnosis is useful in evaluating the severity and localization of peripheral nerve injuries, and the rate and degree of nerve regeneration. Electromyography is helpful to the clinician in detecting, localizing, and following the time course of injury or disease of the anterior horn cells and peripheral nerve, which gives rise to characteristic fibrillation potentials. Electromyography has numerous other applications as a research tool in the study of diseases of neuromuscular transmission and of muscle, and in the study of the effects of electrolytes and drugs, but these applications are unfortunately not yet suited to clinical practice, and are therefore only touched upon in this book, which is designed to be of practical aid to the clinician.

Dr. Licht and his collaborators have presented their material in a clear and concise manner. While their book includes brief chapters on skin resistance, electroencephalography, and electroretinography, it will be of interest primarily to physicians in physical medicine, neurology, and neurosurgery who are concerned with the management of peripheral nerve injury and disease.

David Grob

BOOKS RECEIVED FOR REVIEW

Ageing in Transient Tissues. Ciba Foundation Colloquia on Ageing, Vol. 2. Edited by G. E. W. Wolstenholme and Elaine C. P. Millar. 263 pp., $6.75. Little, Brown & Co., Boston, Mass.

Aids to Embryology, 5th edition. By J. S. Baxter. 196 pp., $2.50. Bailliere, Tindall & Cox, London, England. Distributed by The Williams & Wilkins Co., Baltimore, Md.

Head Injuries and Their Management. By Francis Asbury Echlin. 127 pp., $3.00. J. B. Lippincott Co., Philadelphia, Pa.

Internal Secretions of the Pancreas. Ciba Foundation Colloquia on Endocrinology, Vol. IX. Edited by G. E. W. Wolstenholme and Cecilia M. O'Connor. 292 pp., $7.00. Little, Brown & Co., Boston, Mass.

Management of Emotional Problems in Medical Practice. Edited by Samuel Liebman. 152 pp., $5.00. J. B. Lippincott Co., Philadelphia, Pa.

Medicinal Chemistry, Vol. 3. Edited by F. F. Blicke and R. H. Cox. 346 pp., $10.50. John Wiley & Sons, Inc., New York, N. Y.

Neuropharmacology. Transactions of the Second Conference, May 25, 26 and 27, 1955. Edited by Harold A. Abramson. 328 pp., $4.25. The Josiah Macy, Jr. Foundation, New York, N. Y.

New Bases of Electrocardiography. By Demetrio Sodi-Pallares with the collaboration of Royall M. Calder. 727 pp., $18.50. The C. V. Mosby Co., St. Louis, Missouri.

Polysaccharides in Biology. Transactions of the First Conference, April 27, 28 and 29, 1955. Edited by Georg F. Springer. 271 pp., $5.00. The Josiah Macy, Jr. Foundation New York, N. Y.

Sleep. The Way to Sound and Healthful Slumber. By Marie Stopes. 154 pp., $3.00. Philosophical Library, New York, N. Y.

Studies in Topectomy. Edited by Nolan D. C. Lewis, Carney Landis and H. E. King. 248 pp., $6.75. Grune & Stratton, New York, N. Y.

Surgery of the Hand, 3rd edition. By Sterling Bunnell. 1079 pp., $22.50. J. B. Lippincott Co., Philadelphia, Pa.

Venous Return. By Gerhard A. Brecher. 148 pp., $6.75. Grune & Stratton, New York, N. Y.